AN EVALUATION OF THE ROLE OF AMINO ACIDS IN THE PROCESSING QUALITY OF STORED POTATO TUBERS

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DECLARATION

The author has not been a registered candidate nor an enrolled student for another award of the CNAA or other academic or professional institution during this research programme. Material contained in this thesis has not been used in any other submission for an academic award and is entirely the author's individual contribution. The author has attended appropriate lectures, seminars and conferences in partial fulfilment of the requirements of the degree.

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PUBLICATIONS

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ABSTRACT

This study has been undertaken in order to assess the role of amino acids in determining the processing quality of stored potato tubers. Both the free amino acid content of tubers and possible factors regulating this pool size during prolonged storage were examined, their influence on the processing quality of 2 commercially important cultivars being ascertained over 4 consecutive storage periods.

Turnover of tuber proteins took place throughout storage, the net direction of nitrogen flow depending on the dormancy state of the tuber. As a consequence, free amino acid content was influenced by storage duration. Both cultivars demonstrated an increase of these metabolites during the latter part of storage, caused by an upturn of proteinase activity on the break of dormancy. This was possibly due to *de novo* synthesis of proteinases in particular a 47kDa monomeric enzyme of the aspartic proteinase catalytic class. A high proportion of amides in the free amino acid pool resulted from the activity of glutamine synthetase.

Turnover of proteins increased with storage temperature, although the net direction of nitrogen flux was temperature independent. Consequently, reconditioning regimes were ineffective at lowering free amino acid content, their effects on processing quality being due to the decrease of reducing sugars. When nitrogen flow exhibited a distinct direction this was enhanced by higher temperatures, the late-storage increase of free amino acids demonstrating temperature dependence in Pentland Dell due to greater proteinase activity at 10°C than 5°C. The accumulation of free amino acids, especially the amides, in Pentland Dell at 10°C corresponded with a deterioration of processing quality that could not be accounted for by reducing sugars alone. Despite being in excess with respect to reducing sugars, amino acids had a probable synergistic effect on fry colour production resulting in a darker fry colour per unit reducing sugar.

CONTENTS

ABBREVIATIONS

Abbreviated form	
A.A.	Amino acids
ADP	adenosine diphosphate
A.G.	asparaginase
A.S.	asparagine synthetase
Asn	asparagine
ATP	adenosine triphosphate
CIPC	chlorpropham (isopropyl-3-chlorophenyl carbamate)
DFP	di-isopropylfluorophosphate
DMSO	dimethyl-sulphoxide
E	Molar extinction coefficient
EDTA	ethylenediaminetetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
E64	L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane
Fig.	Figure
FITC	fluorescein isothiocyanate
FPLC	Fast protein liquid chromatography
Frc	Fructose
F.wt. or fresh wt.	Fresh weight
γ–glu hydroxamate	γ-glutamyl hydroxamate
Glc	glucose
Glu	glutamate
Gln	glutamine
GOGAT	glutamine: 2-oxoglutarate aminotransferase
G.S.	glutamine synthetase
HPLC	High performance liquid chromatography
IC_{50}	Enzyme inhibitor concentration producing 50% inhibition
kDa	Kilodaltons

Kav	Distribution coefficient (gel-filtration chromatography)
LAH	lipid acyl hydrolase
n	Sample size
NAD	nicotinamide adenine dinucleotide (oxidised form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NADP	nicotinamide adenine dinucleotide phosphate (oxidised form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
NMR	nuclear magnetic resonance
ODS	Octadecyl (C18) silane column packing material
OPA	o-phthaldialdehyde
PAGE	Polyacrylamide gel electrophoresis
Pi	orthophosphate (inorganic)
PMSF	phenylmethyl-sulphonyl fluoride
Rf	Relative mobility
RFU	Relative fluorescence unit
r.h.	Relative humidity
S.D.	Standard Deviation
SDS	Sodium dodecylsulphate
S.E.	Standard Error
SEC	Size exclusion chromatography
T.A.	transaminases
TBZ	thiabendazole
TCA	trichloroacetic acid
TCNB	tecnazene
TEMED	N,N,N',N'-tetramethylethylenediamine
TLCK	tosyllysine-chloromethyl ketone
TPCK	tosylamide-phenylethyl-chloromethyl ketone
Tris	tris (hydroxymethyl) aminomethane
TRS	Total reducing sugar content

U.S.D.A.	United States Department of Agriculture
UV	Ultra violet
Ve	Elution volume
Vo	Void volume
Vt	Total accessible column volume
(v/v)	Volume to volume
(w/v)	Weight to volume
(w/w)	Weight to weight
$\overline{x}$	Mean

CHAPTER 1. INTRODUCTION

1.1. THE IMPORTANCE OF POTATOES TO MANKIND

1.1.1. The potato as a food crop.

The genus *Solanum* L. contains approximately 2000 species, of which 150 are tuber bearing relatives of the modern cultivated potato *Solanum tuberosum* (Howard, 1970). Although designated as *S. tuberosum*, the modern species is probably a product of cross-breeding between *S. tuberosum* and another species *S. antigenum* (Brouk, 1975). Many of the wild and cultivated potato species in South America are still of immense value as a genetic resource, these being used for breeding beneficial traits such as disease resistance into the modern crop. An example of this use of lesser-known species was the cross-breeding of *S. tuberosum* with *S. demissum* to produce resistance to potato blight *Phytophthora infestans* (Howard, 1970; Brouk, 1975). Although an important food crop in pre-Columbian South America, the potato was not introduced into Europe until around the year 1570. Potatoes became a staple food in Ireland during the 17th century and were first introduced by Irish migrants into North America. However, large scale production did not take place on mainland Europe until the mid-18th century (Brouk, 1975).

Today, the potato tuber is of immense importance to mankind as both a staple foodstuff and fodder crop. Potatoes are the 4th most cultivated crop globally after rice, wheat and maize and are grown throughout the world with the exception of some low-lying tropical regions. In Europe, North and South America it is a staple crop of particular importance in poorer regions. In Great Britain alone, 167,000 hectares of land were planted with the crop in 1991 (Potato Marketing Board, 1992). However, the largest producer of potatoes *per* head of population was Poland (1200kg *per capita*), whilst East Germany and Ireland were also large producers (Brouk, 1975).

1.1.2. The value of the potato in the human diet.

The potato tuber is of relatively minor importance as a source of energy, providing only 10% of the dietary intake in Poland and less than 7% in Ireland (Elton, 1978). Tubers of commercially grown North American cultivars produce between 2.64 and 4.44kJ g^{-1} of fresh tuber tissue, which is small by comparison to other staple sources of carbohydrate such as wheat (hard)13.89kJ g^{-1}, rice (milled white) 15.23kJ g^{-1} and sorghum 14.31kJ g^{-1} (Woolfe, 1987).

However, potatoes are an excellent source of nitrogen providing similar levels of protein (on a dry weight basis) to most cereals (Woolfe, 1987). By comparison to the many leguminous seeds used as key sources of protein in the diet, tuber proteins are present at a relatively low concentration. However, the proteins of the potato tuber are perceived to be of a higher nutritional value (Eppendorfer, Eggum and Bille, 1979; Rhoades, 1982). Potato protein contains relatively high concentrations of the essential amino acids and lysine (Rexen, 1976), although the sulphur amino acids cysteine and methionine are present to a lesser degree than in protein from other staple crops such as wheat and rice (Woolfe, 1987). Due to the high quality of tuber proteins, potatoes are an extremely important source of essential amino acids in the global diet though particularly so in poorer regions. As an example of this key role, in Britain the potato provided 3.4% of household protein intake in 1983 (National Food Survey Committee, 1983). As well as being an important provider of high quality protein, potatoes also account for a significant proportion of dietary fibre intake, this contribution being approximately 15% in Britain (Finglas and Faulks, 1985).

One of the greatest contributions of the potato tuber to the human diet is it's high vitamin and mineral content, in particular that of ascorbic acid (vitamin C). As other staple crops such as wheat, rice, barley and legumes lack this vitamin, the consumption of potatoes as a key food contributes a large proportion of the required ascorbic acid intake, particularly in the diet of developed countries. In addition, tubers

are also a valuable source of B vitamins, chromium, copper, iron, iodine, magnesium, manganese, molybdenum, phosphorus, potassium and zinc (Woolfe, 1987).

To summarise, the importance of the potato as a crop lies mainly in it's high nutritional value, the tubers providing an excellent source of high quality protein and various vitamins and minerals, in particular ascorbic acid. It is because of this nutritional value that the potato has become such a predominant crop, although it's relative importance in the diet varies considerably between regions depending to a large extent on culinary preferences and ease of cultivation.

1.2. COMMERCIAL USES OF THE POTATO

1.2.1. Industrial applications of the potato.

Potatoes have a wide variety of commercial uses in addition to human consumption of the unprocessed tubers. Industrial applications include both starch and ethanol production as a result of the high starch content of tubers. Starch manufacture is particularly important in the Netherlands where over 40% of the potato crop (2.6 million tonnes) was utilised for this purpose in 1979 (Burton, 1986). Potato starch is primarily used in the paper and textile industries. The use of potatoes for ethanol production is of relatively low importance, with the exception of the former Soviet Union, Poland and Germany. Five percent of the Polish crop was utilised for ethanol production in 1979 (Burton, 1986).

1.2.2. The commercial importance of raw potatoes.

In terms of human and animal consumption, potatoes are employed in both the raw and processed form. Raw potatoes are sold for use by both caterers and domestic consumers. In the year May 1990- June 1991, 0.9 million and 2.8 million tonnes were used for these respective purposes in Great Britain, accounting for over 50% of British potato production (Potato Marketing Board, 1992). Of the raw tubers sold for

domestic consumption in Great Britain the vast majority (over 75%) were sold loose (Potato Marketing Board, 1992). However, the market for potatoes has grown increasingly specialised and as a result pre-packed tubers have become increasingly important, particularly in the large retail outlets.

1.2.3. The potato as a processed product.

The use of tubers for processing has become more significant in recent years. The proportion of the United Kingdom crop utilised for processing grew from 19.8% in 1981-82 to 24.1% in 1986-87 (Potato Marketing Board, 1988). Because of this increasing demand for processed potato products, the importation of such goods has increased from 54,000 tonnes in 1965-66 to 500,000 tonnes in 1990-91, and the majority of demand is still met by British production (Potato Marketing Board, 1992).

Potatoes are processed in a variety of ways, including canning, dehydration, freezing and crisp manufacture. Tubers are primarily canned in the form of new potatoes or as small maincrop tubers for the catering trade. In addition, a large proportion are tinned as an ingredient in stews, soups, tinned salads and vegetable mixtures. Only a relatively small fraction of potatoes are processed by canning in Great Britain. In 1984-85 this proportion accounted for 0.2% of domestic consumption (Potato Marketing Board, 1988). The main British cultivars used for canning are the early varieties Arran Comet and Maris Peer.

Dehydration of tubers is mainly performed to produce flakes and granules. These forms of dried potato are subsequently used in the manufacture of a wide variety of reconstituted potato products as well as in a non-reconstituted state as instant mashed potato. Several British cultivars are used for the manufacture of dehydrated products, including the maincrop varieties Maris Piper, Pentland Dell, Pentland Crown, Pentland Squire and King Edward (Potato Marketing Board, 1988). Due primarily to the ease of storage, canned and dehydrated potato products are of great importance to the

catering industry in Great Britain, making up 30% of non-domestic consumption in 1990-91. In contrast, domestic utilisation of these products is low forming only 5% of usage in 1990-91 (Potato Marketing Board, 1992).

Processed potatoes are frozen mainly in the form of chips or French-fries, although other forms of frozen potato, such as part-cooked roast potatoes and reconstituted products, have become increasingly important. In Great Britain, the various forms of frozen product made up 48% and 51% of processed tuber usage by domestic and catering consumers respectively in 1990-91. Frozen products used approximately 17% of total ware tuber production (including imports) in this period (Potato Marketing Board, 1992). Frozen potato products are more important in the USA, accounting for approximately 25% of tuber production by 1970 (Burton, 1986). The main varieties utilised for the production of frozen French-fries in Great Britain include the maincrop cultivars Pentland Dell, Pentland Crown, Pentland Hawk, Pentland Squire and Maris Piper and also the earlier variety Wilja. In addition, the North American cultivar Russet Burbank is becoming increasingly significant in United Kingdom markets (Potato Marketing Board, 1988).

The other major processing use of tubers is the manufacture of potato crisps (known as chips in the USA). This is one of the oldest forms of commercial tuber processing, although in 1939 only approximately 1% of tubers were utilised for this purpose in the USA (Talburt, 1975). However, production of crisps has become of major commercial importance in the post-war period both in the USA and Great Britain. By 1990-91 crisps constituted 11% of total potato consumption in Great Britain (Potato Marketing Board, 1992). The major cultivar used in British crisp manufacture is Record, although earlier varities such as Home Guard and Wilja are used to a lesser extent (Potato Marketing Board, 1988).

1.2.4. Other commercial uses.

In addition to the human consumption of tubers in either a raw or processed form, over 10% of British potato production is used as either animal fodder or discarded (Potato Marketing Board, 1992). Much of the potato-based stockfeed sold commercially is produced as a bi-product of the processing industry. Tubers are also sold commercially as seed, and less than 1% of production was exported for this purpose in 1990-91 (Potato Marketing Board, 1992).

1.3. PROBLEMS OF POTATO STORAGE

1.3.1. The need for potato storage under controlled conditions.

Potatoes are consumed throughout the year in both raw and processed forms and the level of consumption varies only slightly from month to month (Potato Marketing Board, 1992). To cope with this constant demand it may be necessary to store tubers for long periods following harvest, possibly up to 9 or 10 months. In Britain, tubers are primarily stored indoors either unseparated or in pallet boxes. The temperature and humidity of the store is regulated in order to maintain the crop quality at as high a standard as possible over the required period. The temperature regime varies with cultivar, condition on harvest and in store and the type of product for which it will be used.

1.3.2. Curing and it's associated problems.

Before entry into long term storage, tubers are often cured for a short period of time (usually approximately 2 weeks) in order to enable the tuber skins to finish setting (periderm formation). This process generally involves maintaining tubers at 12-15°C and a relative humidity (r.h.) of around 95% (Potato Marketing Board, 1992). The relatively high temperature allows the rapid healing of any wounds incurred before, during and after harvest. Periderm formation and wound healing are of prime

importance to successful storage as they reduce moisture loss and prevent infection by either bacterial or fungal pathogens.

One drawback of curing tubers at both a high temperature and relative humidity, is that pathogens already present both on the tuber surface and within the tuber tissue will be able to multiply at a high rate. In order to control diseases such as bacterial soft rot (*Erwinia* spp.) and the surface blemishing fungal disease, Silver scurf (*Helminthosporium solani*), it is often necessary to cure tubers at below 90% r.h. Fungal infection is also reduced in storage by post-harvest treatment of the crop with fungicides (Meijers, 1987; Hide, 1992). These include thiabendazole (TBZ) for the control of dry rot (*Fusarium* spp.), gangrene (*Phoma foveata*), skinspot (*Polyscytalum pustulans*) and silver scurf, and also tecnazene (TCNB), a sprout suppressant which additionally controls dry rot and to a lesser extent gangrene, skinspot and silver scurf (Potato Marketing Board, 1992). Both the above chemicals may be applied to the crop in the form of a spray or powder, although TCNB is primarily applied as granules and may also be formulated for treatment of the crop by fogging (Potato Marketing Board, 1992). No chemical treatments are available for the treatment of the bacterial infections that cause soft rot (Meijers, 1987).

1.3.3. General storage problems.

Following curing, the storage temperature of tubers is reduced to the holding temperature. This is determined by a variety of factors including the anticipated use of the tubers, likely storage duration and condition of the crop. To bring down the temperature of a large bulk of tubers takes several days and the tubers may still undergo curing in the early part of this temperature reduction process. As a result it is often necessary to shorten the curing period to account for this additional curing effect.

Different storage problems are experienced at high and low holding temperatures. High temperatures have many drawbacks. At over 10°C, weight loss may be a serious problem resulting in softening of the tuber, reducing the both the value and usefulness of the crop. In addition, sprouting may reduce the tuber weight as the loss of water is greater through sprout tissue than through the tuber skin (Burton, 1966). The growth of sprouts also utilises tuber carbon and nitrogen reserves resulting in weight loss and a reduction in the nutritional value of the tuber. Many tubers can also be lost at high temperatures through bacterial and fungal infections such as soft rot and silver scurf (Potato Marketing Board, 1992). Sprouting of tubers at high storage temperatures may be reduced by the use of sprout supressant chemicals, including chlorpropham (isopropyl-3-chlorophenyl carbamate) and TCNB (1,2,4,5-tetrachloro-3-nitrobenzene). Chlorpropham (CIPC) is the most commonly used sprout suppressant and acts through the inhibition of cell division. As a result, CIPC is usually applied to tubers in store after the curing process has been completed. Application is predominantly by fogging, granular formulations being less effective due to the difficulty of obtaining an even dosage on a large bulk of tubers (Potato Marketing Board, 1992). Low storage temperatures of below 4°C, may also cause a reduction in tuber quality. Infection with the storage diseases gangrene and skinspot may be increased and in addition, tubers are more susceptible to cell damage resulting in black spot, a form of blue-black discolouration of tissue beneath the skin (Meijers, 1987; Potato Marketing Board, 1992).

The store holding temperature may be first achieved and then maintained by a variety of methods. For short-term storage of small volumes of tubers, convection ventilation may be used. For cooling by this method, air enters through the base of the potato stack and exits through the top of the store. This process may be made efficient enough to be applied to larger volumes of tubers through the use of forced-draught ventilation, automatically controlled fans providing a flow of cool air through the tuber stack. Condensation within the store may be reduced by the recirculation of air,

which also removes temperature differences within the stack. However, for long-term storage, refrigeration is used as the primary means of temperature control, often in conjunction with air recirculation (Potato Marketing Board, 1992).

The anticipated use of a crop dictates not only the holding temperature but also the storage duration. Many of the early varieties are consumed soon after harvest in the form of new potatoes and as a result these are only stored for relatively short periods commonly at 5°C (Potato Marketing Board, 1988). A year-round demand for new potatoes is fulfilled by imports and canning, which removes the need for long-term storage regimes.

1.3.4. Prolonged storage of tubers for processing.

Maincrop ware potatoes may be stored for long periods and this poses many problems, especially if tubers are to be used for processing. In addition to the general storage problems such as sprouting and disease control, the tuber reducing sugar content is of crucial importance if tubers are to be processed, particularly in the form of chips, crisps or other fried potato products (Smith, 1975). Reducing sugars and amino acids are the primary reactants in the Maillard reaction (Maillard, 1912; Hodge, 1953; Mauron, 1981), a complex series of non-enzymic reactions that result in the formation of brown pigmentation upon cooking (detailed in section 1.4.). The processing of tubers containing high concentrations of these reactants results in the production of chips or crisps with a dark fry colour and bitter taste that are unacceptable to the consumer (Roe, Faulks and Belsten, 1990). Consequentially, it is in the best interest of the processor to maintain the crop under storage conditions that will limit any upturn of reducing sugar content during storage, whilst also maintaining tuber dormancy for as long a period as possible. The tuber reducing sugar content on entry into storage is affected by the cultivar, weather, site, season and physiological maturity. Immature tubers display a high sucrose content on lifting, subsequently resulting in an accumulation of reducing sugars (Burton, 1969; Sowokinos, 1978).

The temperature and duration of storage also have a profound effect on the reducing sugar content. Tubers stored at a holding temperature of 5°C or below generally exhibit a rapid rise in reducing sugars (Fig. 1.01.) (Müller-Thurgau, 1882 cited by Van Es and Hartmans, 1987; Barker, 1932; Burton, 1966; Burton, 1969; Isherwood, 1973; Coffin, Yada, Parkin, Grodzinski and Stanley, 1987). This undesirable increase, as a consequence of starch and sucrose degradation in the tuber, is termed low-temperature sweetening. It is thought that this process may be partially due to decreased respiration at low storage temperatures, this enabling reducing sugars to accumulate (Tishel and Mazelis, 1966). An alternative theory is that temperature influences the activities of certain key enzymes of sugar metabolism. The key enzyme of sucrose hydrolysis, invertase, is cold activated, whereas phosphofructokinase, an enzyme regulating the entry of sugars into glycolysis is cold labile (Van Es and Hartmans, 1987).

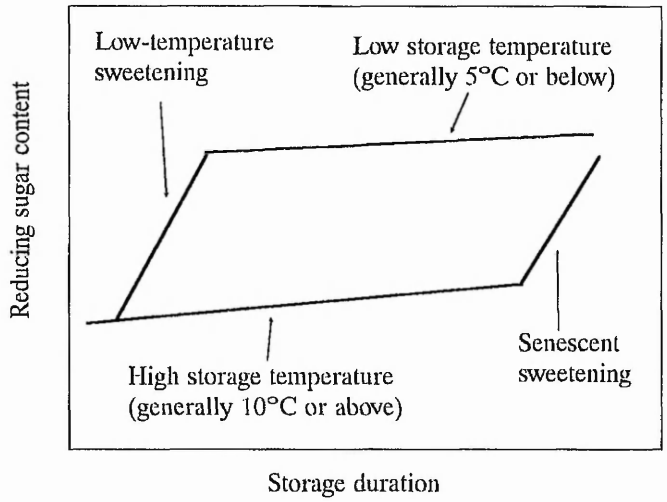


Figure 1.01. The influence of storage temperature and duration on tuber reducing sugar content.

The accumulation of reducing sugars as a consequence of low-temperature sweetening may be partially reversed by a short-term increase in storage temperature.

This temperature treatment at 15-20°C is usually performed over a period of up to 6 weeks, and is termed reconditioning. It is thought that reconditioning treatments may lower the reducing sugar concentration by causing either a burst in respiration or reconversion back into starch (Heulin and Barker, 1939; Schippers, 1977; Williams and Cobb, 1992, 1993).

Tubers stored at high holding temperatures of 10°C and above may also display an increase in reducing sugar content (Fig. 1.01.), this generally occurring after a prolonged period in storage (Van Vliet and Schriemer 1963; Burton, 1966; Van Es and Hartmans, 1987). This rise, known as senescent sweetening, is accompanied by starch and sucrose degradation. It has been proposed that membrane breakdown caused by senescence may be a factor enabling the leakage of reducing sugars from the amyloplast (Van Es and Hartmans, 1987). The increase of membrane permeability within the cell, may also remove the compartmentation of starch from the enzymes of hydrolysis (Workman, Cameron and Twomey, 1979). Because of the senescent nature of this upturn in reducing sugars, reconditioning is ineffective as a means of lowering their concentration later in storage.

Due to the above temperature related problems in the prolonged storage of tubers for processing, holding temperatures in the region of 7-8°C are usually used for those utilised for chip production (Potato Marketing Board, 1988). In the case of Record tubers used in crisp manufacture, a temperature of 9-10°C is more common. If tubers contain a high concentration of reducing sugars, the acceptability of the processed product may be improved by blanching of the chips or crisps in hot water prior to frying (Smith, 1977). This blanching process leaches sugars from the tuber tissue resulting in a lighter fry colour. However, the procedure has disadvantages in that it may adversely effect the texture of the product and the leachate creates environmental problems in it's disposal.

1.4. THE MAILLARD REACTION

1.4.1. The importance of the Maillard reaction.

The Maillard or non-enzymic browning reaction was first recognised by the French chemist Louis Maillard (1912), upon observing the formation of brown pigments caused by heating a solution of glucose and lysine. It is a reaction of great importance in the preparation and processing of a variety of foods, producing not only pigmentation but also flavours and aromas (Table 1.1.).

Table 1.1. Classification of Maillard flavours.
(Adapted from Hodge, Mills and Fisher, 1972)

Reaction product	Flavour or aroma
Nitrogen heterocyclics eg pyrazines, thiazoles and pyridines	Nutty, roasted, baked aromas
Cyclic enolones eg maltol and isomaltol	Caramel aromas
Polycarbonyls eg pyruvaldehyde	Burnt, pungent aromas
Monocarbonyls eg Strecker aldehydes	Aldehydic, ketonic aromas

The reaction products are predominantly found in food and beverages heated during preparation, including bread, soy protein foods, cooked potatoes, meats, fish, vegetables, coffee, cocoa and beer (Mauron, 1981; Namiki, 1988). Whilst the properties of Maillard reaction products are desirable in a variety of foodstuffs, in several products such as dried milk, concentrated fruit juices and processed potatoes they lead to an unacceptably dark colour and bitter taste (Ames, 1988; Roe *et al*, 1990). The browning reaction may also have an adverse effect on the nutritional value of foods by decreasing the availability of amino acids and reducing protein digestibility (Mauron, 1981). This is of special significance in processed potato products as the potato is such an important source of essential amino acids, particularly lysine. The lysine Maillard products monofructose-L-lysine and difructose-L-lysine are not

absorbed efficiently by the intestine. Any Maillard products that are absorbed are not utilised for protein synthesis (unlike lysine) and are mainly excreted (Sherr, Lee and Jelesciewicz, 1989).

The first coherent explanation of the non-enzymic browning process was put forward by Hodge (1953), the reaction being shown to consist of many complex stages. Mauron (1981) simplified this by dividing the Maillard reaction into 3 stages termed early, advanced and final reactions. The overall rate of colour formation and degree of browning depends on several factors including temperature, pH and the initial reactants (Namiki, 1988).

1.4.2. Early reactions.

The first step in the Maillard reaction involves condensation of the carbonyl group of a reducing sugar or other carbonyl compound and the free amino group of an amino acid, peptide or protein. The resultant condensation product is converted into a Schiff's base by the loss of a water molecule and subsequently into a N-substituted glycosylamine by cyclisation (Fig. 1.02.). These reaction steps are fully reversible as the N-substituted glycosylamine may be hydrolysed under aqueous conditions to produce the original reactants (Hodge, 1953). In the case of N-substituted glycosylamines derived from amino acids, these are converted immediately into 1-amino-1-deoxy-2-ketoses by a process known as the Amadori rearrangement (Fig. 1.02.). This step involves the transition from an aldose to ketose sugar derivative, a conversion catalysed by weak acid conditions, amino acids themselves acting as catalysts for this reaction (Namiki, 1988). In strong acid or water solutions the Amadori rearrangement may not proceed as the N-substituted glycosylamine is hydrolysed back to the primary reactants (Hodge, 1953).

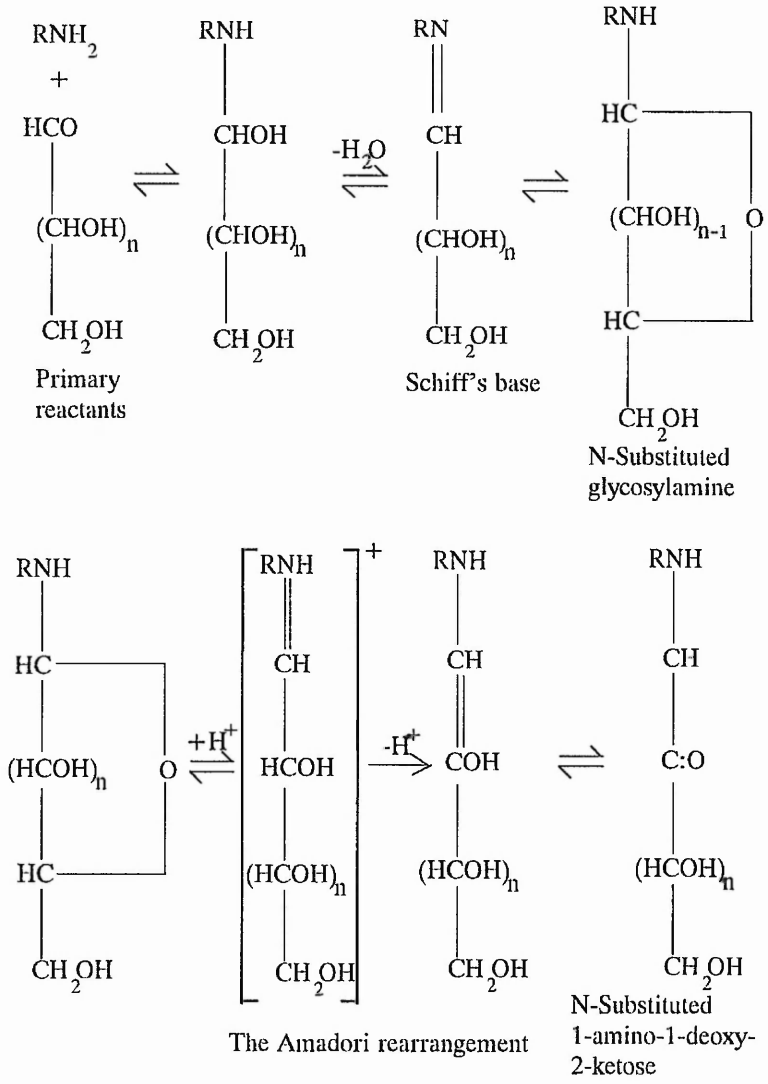


Figure 1.02. The early stages of the Maillard reaction, including the initial condensation reaction of an aldose and free amino compound and the Amadori rearrangement. (From Hodge, 1953).

These early reaction steps do not cause either browning or flavour production and in some foods, the reaction may not progress further if the processing temperature is low or the reaction time short. This is due to the relative stability of the products of the Amadori rearrangement (N-substituted 1-amino-1-deoxy-2-ketoses). Higher processing temperatures favour further reactions that eventually lead to colour and flavour production, these advanced Maillard reactions initiate either directly or indirectly from the Amadori rearrangement products.

1.4.3. Advanced reactions.

The Amadori rearrangement products may undergo a complex series of reactions (Fig. 1.03.). At low pH (pH 7.0 or below) 1,2-enolisation may take place, followed by the loss of a hydroxyl group, deamination, water addition and dehydration steps (Mauron, 1981, Namiki, 1988). These reactions result in the formation of either furfural or hydroxymethylfurfural from pentose and hexose reducing sugars respectively (Ames, 1988). At higher pH (above pH 7.0), 2,3-enolisation may occur, followed by the irreversible elimination of the amino group from C1 and subsequent reactions to yield fission products. These include short chain carbonyls, dicarbonyls and reductones (Hodge, 1967; Mauron, 1981; Ames, 1988; Namiki, 1988).

Both of these enolisation processes may take place in the same system and the products are produced in varying ratios depending on the pH. The resultant compounds are highly reactive and incorporation of amino groups may take place, causing the production of a variety of heterocyclic compounds with colour and flavour properties. As an example, furfural from the 1,2-enolisation process may react with amino compounds to produce pyrrole and pyridine derivatives which convey a nutty or roasted flavour to the food. Heterocyclic compounds may also be formed by a process known as the Strecker degradation. In this reaction, dicarbonyl compounds from the above enolisation pathways react with amino acids to form aldehydes and α-aminoketones. A condensation reaction involving two α-aminokenone molecules results in the production of pyrazines. N, S and O-heterocyclic compounds have different flavour properties. The N and S-heterocyclics such as oxazolines, pyrazines, pyrroles, pyrrolidines, pyridines and thiazoles convey the nutty, roasted flavour of a variety of foodstuffs including potato products, whereas O-heterocyclic compounds such as maltol and isomaltol are responsible for caramel aromas (Mauron, 1981).

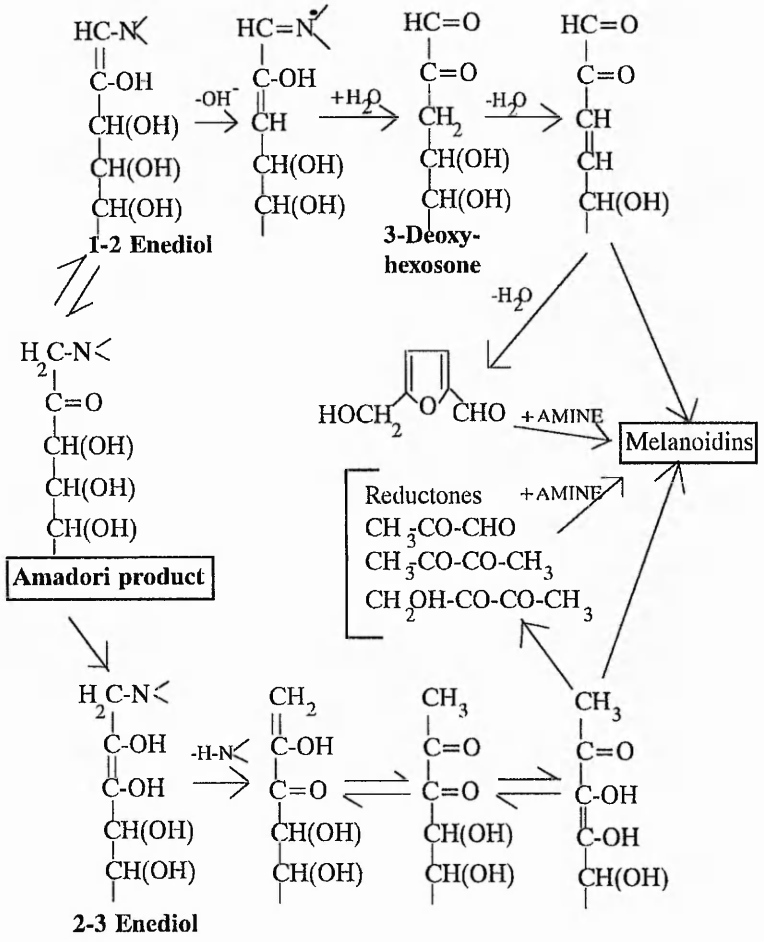


Figure 1.03. The enolisation and degradation of Amadori products to produce flavoured and coloured compounds. (From Hodge, 1967).

In addition to the formation of low molecular weight flavour compounds, further reactions take place to produce the melanoidin pigments responsible for the brown colour formation in a variety of cooked foodstuffs.

1.4.4. Final browning reactions and the properties of melanoidins.

The brown melanoidin pigments are formed by the polymerisation of compounds produced in the advanced reaction stages (eg. unsaturated carbonyls and furfural) in the presence of amine (Fig. 1.03.) (Mauron, 1981). Melanoidins are relatively inert molecules with molecular weights of over 1 kDa and a complex structure containing furan rings. This structure varies depending on the reactants (reducing sugars and

amino acids etc.) and reaction conditions (pH, reaction temperature and reaction time), higher reaction temperatures and longer heating times increasing the carbon: nitrogen ratio and the degree of unsaturation and aromaticity (Ames, 1988). It is thought that the brown colour of these pigment molecules is due to the presence of heterocyclic amines (Mauron, 1981).

1.4.5. Primary Maillard reactants as a measure of potential browning in processed potato tubers.

Free amino acids are generally present at higher concentrations in tubers than reducing sugars, the latter reactants thus being assumed to limit the degree of browning (Dahlenberg, 1982; Marquez and Añon, 1986). As a result, the tuber reducing sugar content has been adopted by the processing industry as a measure of potential colour formation, and this is determined primarily by enzyme assay (Smith, 1977). However, some studies have shown tubers grown under high nitrogen fertilisation regimes to develop dark fry colours on processing, these not being entirely attributable to reducing sugar concentration (Hughes and Fuller, 1984; Roe *et al*, 1990). This suggested that free amino acid content may also be a factor controlling the degree of browning, although the importance of free amino acids may be secondary to that of reducing sugar content (Habib and Brown, 1957; Hope, MacKay and Townsend, 1960; Roe *et al*, 1990).

1.5. AIMS OF THE INVESTIGATION

Amino acids are Maillard reactants and therefore crucial to processing. However, a paucity of information exists in the literature concerning tuber amino acid metabolism and how it is affected by storage duration and regime. As a consequence, it is an aim of this investigation to examine the free amino acid content of commercially important cultivars throughout prolonged storage. It is envisaged that the experimental storage of tubers at the upper and lower temperature limits used in commercial stores, will

enable the identification of storage factors that control either the concentration or composition of these Maillard reactants. Possible metabolic processes involved in the control of the free amino acid pool will also be studied through the use of enzyme assay techniques. This study will allow a coherent model of storage amino acid and protein metabolism to be developed and as a consequence, demonstrate how different storage regimes affect the free amino acid acid pool.

Information in the literature regarding the importance of free amino acids as factors influencing fry colour is sparse and somewhat contradictory. As a result, it is an objective to assess critically the relative importance of free amino acids and reducing sugars in determining fry colour intensity throughout storage. This should enable the true significance of the free amino acid content to the processing potential of stored tubers to be established. It is hoped that in conjunction with the effects of storage duration and regime on Maillard reactant contents, the relative influence of free amino acids on fry colour, will permit optimum storage conditions and removal time for processing to be estimated.

Reconditioning treatments are used extensively for the recovery of tuber processing potential during storage by the lowering of reducing sugar content. It is an aim to examine the effect of these treatments on free amino acid metabolism and pool size. As a result of this study's findings and the degree of amino acid influence on fry colour, the relative benefits and drawbacks of reconditioning regimes to the processing quality of stored tubers will be assessed.

CHAPTER 2. AMINO ACIDS AS SUBSTATES FOR THE MAILLARD REACTION IN STORED POTATO TUBERS

2.1. INTRODUCTION

Total reducing sugar (TRS) content has long been used as a marker for tuber processing potential by potato processors. Although variation of fry colour during storage may usually be attributed to reducing sugar pool size due to the relatively excessive concentration of amino acids (Marquez and Añon, 1986), anomalies in the relationship of reducing sugars to fry colour do occur. In addition to reducing sugars, amino acids are also substrates for the Maillard Reaction (Hodge, 1953) and possibly also determinants of fry colour (Wünsch and Schaller, 1972; Burton, 1982; Cobb, Hart and Storey, 1990; Roe, Faulks and Belsten, 1990). Hughes and Fuller (1984) and Roe *et al* (1990) observed that potato tubers grown under high nitrogen conditions produced more non-enzymic browning per unit of reducing sugar than those grown with low levels of nitrogen fertiliser. Due to the link between the rate of nitrogen fertiliser and tuber amino acid content (Hope, Mackay and Townsend, 1960; Hughes and Fuller, 1984; Millard, 1986; Roe *et al*, 1990), it was indicated that amino acid content is of possible importance in determining processing potential.

Previous studies on the amino acid content of tubers during prolonged storage have shown varying trends. Talley, Fitzpatrick and Porter (1964), Talley, Toma and Orr, (1984) and Talley and Porter (1970) observed little change in pool size as a result of prolonged storage, whilst Hart, Pallett and Cobb (1986) observed an initial rise subsequently followed by fluctuation over the first 12 weeks of tuber storage. Studies of the amino acid composition of stored potato tubers have shown the free amino acid pool to contain a high proportion of the amides asparagine and glutamine (Talley *et al*, 1964; Talley and Porter, 1970; Davies, 1977; Synge, 1977; Millard, 1986; Hart and Cobb, 1988). Results obtained by Hart and Cobb (1988) also suggested

conversion of glutamine to asparagine throughout storage possibly due to the action of asparagine synthetase. Asparagine possibly has a role in determining fry colour intensity in conjunction with that of reducing sugars. Reconditioning of tubers at higher temperatures for short periods decreases reducing sugar content (Fitzpatrick and Porter, 1966; Burton and Wilson, 1978; Burton, 1982) and as a result improves fry colour, athough relatively little is known about the effect of this treatment on the amino acid pool size and composition.

In this study over four storage seasons, the total free amino content and the individual amino acids asparagine, glutamine, aspartate and glutamate have been measured throughout prolonged storage in tubers of the commercially important varieties Pentland Dell and Record at 5° and 10°C. In all seasons tubers were stored for up to 40 weeks at both temperatures in order to examine the effect of storage duration on amino acid content and composition, this storage period being at least as long as that used in commercial operations. Due to the effect of storage temperature on reducing sugar content (Burton, 1982) it was necessary to examine any possible temperature influence on amino acid content. Temperature effects on fry colour intensity were thus explained in terms of the concentrations of these metabolites. The effects of short-term reconditioning at 20°C on amino acid content were also examined, thus explaining the effectiveness of reconditioning in improving fry colour in terms of both reducing sugars and amino acids.

In all four storage seasons the effects of total free amino acid pool size on fry colour intensity were assessed critically, thus showing the importance of free amino acids relative to reducing sugars as markers for the processing potential of stored tubers. The individual amide contents were determined throughout storage in order to further investigate any possible conversion of glutamine to asparagine, these individual amides also being examined as possible key determinants of fry colour.

2.2. MATERIALS AND METHODS

2.2.1. Storage procedure.

Tubers of both the cultivars Pentland Dell and Record were harvested in October in all 4 seasons studied, and transported to the Potato Marketing Board Experimental Station at Sutton Bridge, Lincolnshire, U.K.

Both cultivars underwent an initial curing period of between 1 and 2 weeks at 15°C (95% r.h.) in a controlled environment room (Table 2.1.), to promote wound healing and periderm formation. Following curing, the tubers of both cultivars were divided into wooden boxes for storage in controlled environment rooms at 5° and 10°C (95% r.h.) and subsequently held at these storage conditions for up to 40 weeks.

Table 2.1. Harvesting and curing schedule.Pentland Dell.

Storage season	Harvest date	Curing period (days)	Date into store
1989-1990	16/10/1989	14	30/10/1989
1990-1991	10/10/1990	12	22/10/1990
1991-1992	14/10/1991	15	29/10/1991
1992-1993	7/10/1992	14	21/10/1992

Table 2.2. Harvesting and curing schedule. Record.

Storage season	Harvest date	Curing period (days)	Date into store
1989-1990	16/10/1989	14	30/10/1989
1990-1991	8/10/1990	14	22/10/1990
1991-1992	21/10/1991	8	29/10/1991
1992-1993	16/10/1992	12	28/10/1992

Tubers were treated with the sprout suppressant chlorpropham (CIPC) as required during storage to minimise sprout growth. The sprout suppressant CIPC may be absorbed into the wood of tuber storage boxes so reducing the effectiveness of the treatment. To avoid this problem, in the 1992-93 season these boxes were replaced by plastic trays.

Potatoes were sampled for analysis at 4-weekly intervals during storage with the exception of the 1989-1990 storage season, in which sampling was carried out at approximately 6 week intervals. In addition to the 5° and 10°C storage temperature regimes, tuber batches were subsampled from these temperatures during storage and placed into a controlled environment room at 20°C (95% r.h.) for up to 6 weeks, in order to recondition the crop. This reconditioning procedure (Fig 2.01.) was carried out at early, mid and late stages during the 40 week period, with the exception of the 1989-1990 storage season. In this first season of the study, tubers were reconditioned for 4 week periods at only mid and late-storage (Fig 2.01.). After each reconditioning period at 20°C the tubers were sampled for analysis. In the 1991-1992 season tubers were sampled after 2 weeks in addition to the full term of 6 weeks reconditioning. Tubers were treated with granular CIPC on transfer from 5° or 10°C to the reconditioning temperature in order to minimise sprout growth.

2.2.2. Sampling procedure.

On each sampling occasion, 5 tubers of both cultivars were randomly selected from the 5° and 10°C storage rooms. These tubers were packed in insulated cold boxes containing vermiculite equilibrated at the storage temperature. This inert granular material provided protection from mechanical damage as well as extra insulation during transit to The Nottingham Trent University by car. Tubers were used for immediate extraction of amino acids, soluble proteins and reducing sugars.

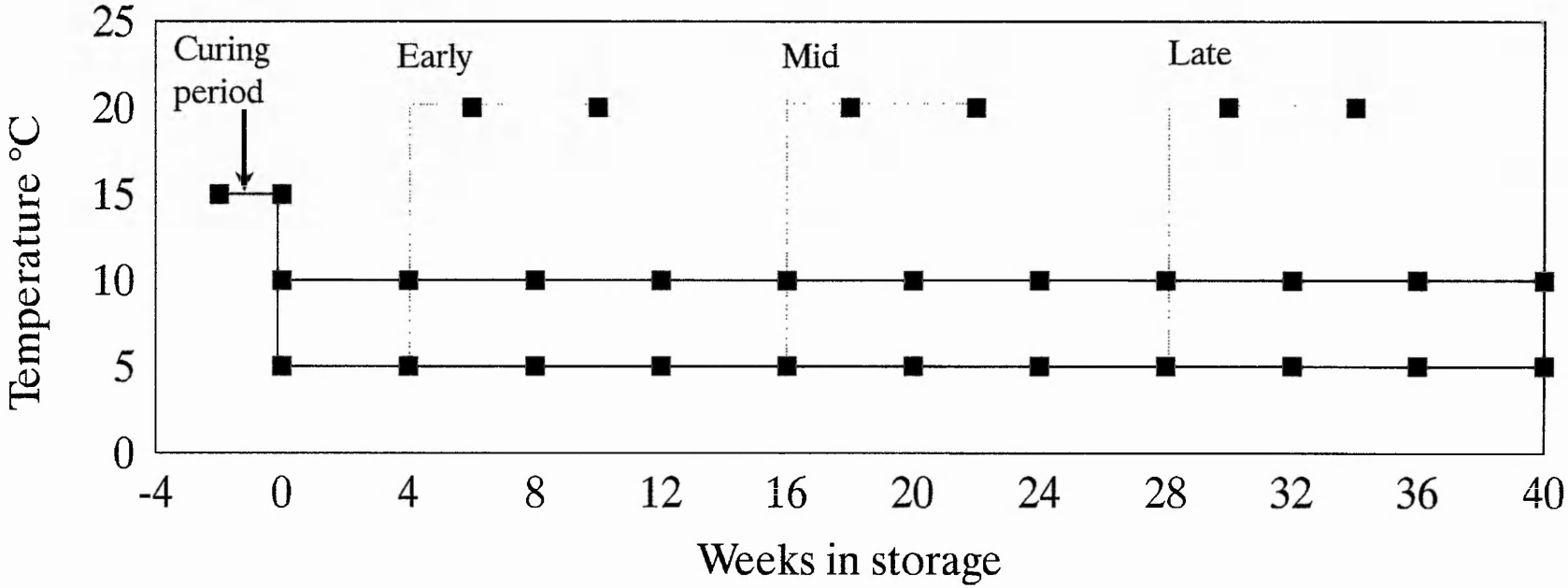


Figure 2.1. Storage and sampling scheme for a typical season. Tubers of both cultivars were stored at 5°C, 10°C(—) and reconditioned at 20°C(-). Sampling was performed at the above points (■) in storage.

2.2.3. Extraction procedure.

Longitudinal cores (10mm diameter) were removed from the centre of each tuber and divided into three equal sections: basal, median and apical (Fig 2.02.).

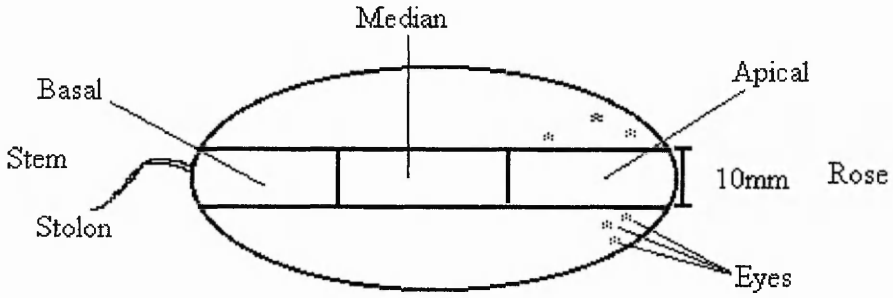


Figure 2.02. Division of the longtitudinal tuber core into three equal sections.

In all cases, basal sections (stem end of the tuber) were used for extraction of metabolites by a modified version of the method of Hart (1986). Each basal core section was weighed in 5ml 100% (v/v) ethanol (SpectrosoL, BDH), homogenised with 5ml cold pure water (Millipore, Milli Q reagent grade water) and centrifuged at 3000 r.p.m. for 5 min at 0°C (Denley model BR401 refigerated centrifuge or MSE Chilspin). The supernatant was stored on ice in a glass freeze-drying vial and the pellet resuspended in 3ml cold pure water. This suspension was then centrifuged at 3000 r.p.m. for 5min at 0°C and the supernatant pooled with that from the previous spin. The pellet was resuspended in a further 2ml cold pure water and centrifuged as above, the supernatant being pooled with that already obtained. The pooled supernatant was frozen at -70°C. Frozen tuber extracts were then freeze-dried over 48 hr using an Edwards, Minifast model 680 shelf freeze-dryer, and then stored at -70°C until required.

2.2.4. Determination of tuber tissue total free amino acid concentration.

Total primary free amino acid content of tuber tissue was determined by ninhydrin assay (Hart *et al*,1986). Four mg of freeze-dried tuber extract was dissolved in 10ml pure water (Millipore, Milli Q reagent grade water) using gentle agitation and 1ml of this solution mixed with 1.2ml ninhydrin reagent and 0.8ml hydrazine reagent. The contents of these reagents were as follows; **Ninhydrin reagent**: 3.3g ninhydrin (Sigma) in 240ml 2-methoxyethanol (GPR, BDH), 50ml 4M aq. acetate containing 25g sodium acetate trihydrate (Sigma) and 5ml glacial acetic acid (AnalaR, BDH), and in addition 15ml glacial acetic acid (AnalaR, BDH), the total volume made up to 500ml with pure water. **Hydrazine reagent**: 52mg hydrazine sulphate (Sigma) in 250ml pure water with one drop of conc. sulphuric acid (Rectapur). The tuber extract-reagent mixture was boiled for 15min and then allowed to cool before 3ml 50% (v/v) ethanol (diluted from SpectrosoL, BDH) was added.

After standing for 10min the absorbance was read at 570nm using a Perkin-Elmer model 550S UV-visible spectrophotometer against a distilled water-reagent blank. Absorbance readings were converted to amino acid concentration using a regression coefficient derived from a glycine (Sigma) standard curve, values being expressed as mg (amino acid) g^{-1}(freeze-dried powder). Results were finally expressed as mg (amino acid) g^{-1}(tuber tissue fresh weight) using a conversion factor (weight of freeze-dried extract / fresh weight of tissue used for extraction).

2.2.5. Determination of individual tuber tissue free amino acid concentrations.

Concentrations of key individual free amino acids were determined throughout each season by reverse-phase HPLC using Beckman System Gold equipment (Fig 2.03.) consisting of a model 507 autosampler, model 116/126 programmable pump unit and a model 157 fluorescence detector. Data from this detector was converted from

analogue to digital by a model 406 interface and stored on an Amstrad personal computer utilising Beckman System Gold software (version 4.03).

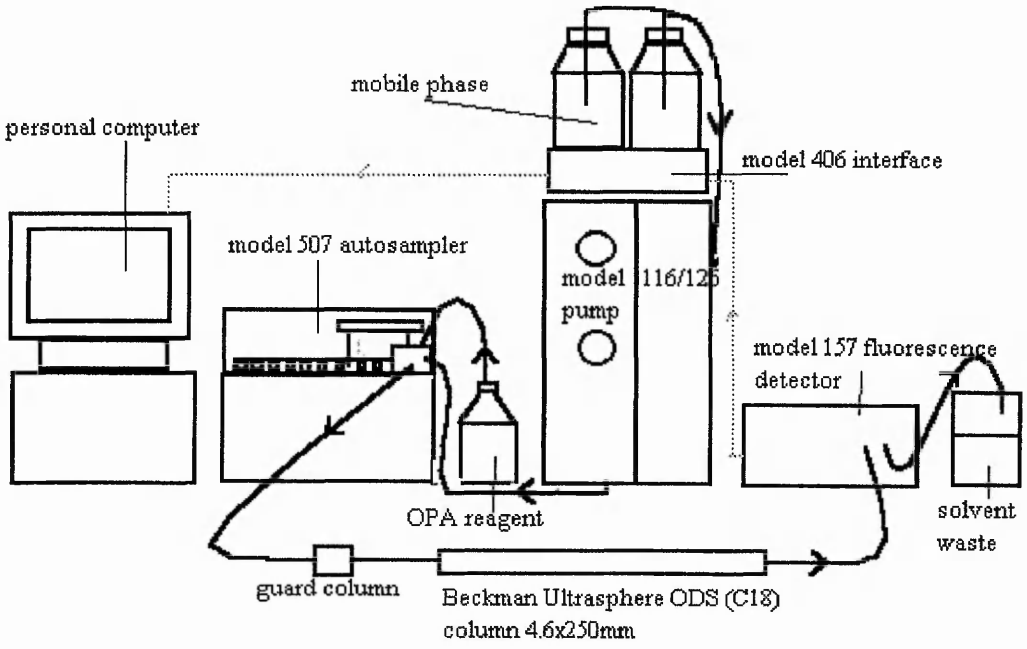


Figure 2.03. Beckman System Gold HPLC fitted for primary amino acid analysis using fluorescence detection.

Reverse-phase HPLC, as with other forms of HPLC, involves the use of a column containing a stationary phase to separate the solutes (eg. amino acids) that are carried within a liquid mobile phase. With reverse-phase HPLC the polarity of the liquid mobile phase is higher than that of the stationary phase, the mobile phase generally being of an aqueous nature containing an organic solvent such as acetonitrile. With most reverse-phase columns the stationary phase consists of silica gel particles (3-18μm diameter) containing many minute pores increasing their surface area. These particles are coated with a bonded surface of hydrocarbon chains (eg. C8 or C18) so producing a non-polar surface.

As during a reverse-phase separation the more polar solutes have a greater affinity for the mobile phase, the solutes are eluted from the column in order of decreasing polarity. Retention times of solutes may be changed by altering the composition and hence the polarity of the mobile phase, a technique known as gradient elution. A lowering of the mobile phase polarity by an increase in the organic solvent proportion will decrease solute retention time.

For the analysis of tuber amino acids, the Beckman model 507 autosampler was fitted with a reagent addition cassette. This attachment enabled automated pre-column derivatisation of primary amino acids with OPA(ortho-phthaldialdehyde)-2-mercaptoethanol reagent (Lindroth and Mopper,1979). The reagent contained 54mg OPA (Sigma) dissolved in 1ml methanol (Fisons, HPLC Solvent). To this was added 9ml 400mM borate buffer pH 9.5 (Bates and Bower,1956 cited by Dawson, Elliott, Elliott and Jones, 1969) and 0.2ml 2-mercaptoethanol (Sigma). The borate buffer was made up as follows: 50ml 25mM di-sodium tetraborate decahydrate (BDH, AnalaR; 9.525gl^{-1}) and 8.8ml 100mM NaOH (BDH, AnalaR; 4gl^{-1}) diluted to 100ml with pure water. The OPA-2-mercaptoethanol reagent reacts with primary amino acids to produce highly fluorescent derivatives (Fig 2.04.), these are 1-alkyl-thio-2-alkyl substituted isoindoles that are detected using an excitation wavelength of 340nm and emission wavelength of 455nm.

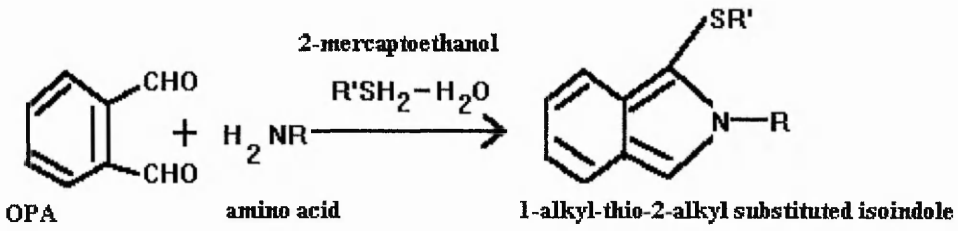


Figure 2.04. The derivatisation of primary amino acids with OPA-2-mercaptoethanol reagent. (From Blankenship, Krivanek, Ackermann and Cardin, 1989).

Millipore, Milli Q reagent grade water was used throughout and all buffers filtered through 0.22µm membranes (Millipore) to remove larger impurities that might block narrow tubing. To prepare each sample, 4mg of freeze-dried tuber extract was dissolved in 10ml 50mM HCl (diluted from AnalaR 1M HCl, BDH) containing 250µM homoserine (29.775mg l^{-1}, Sigma) and then diluted by a factor of 100, so that each sample contained a 2.5µM homoserine internal standard. Samples were loaded into vials and placed on the autosampler carousel ready for derivatisation and injection.

Upon the initiation of each sample run the autosampler needle withdrew 25µl of extract from the autosampler vial which was mixed with an equal volume of OPA-2-mercaptoethanol reagent in the reagent addition cassette. The sample-reagent mixture was allowed to react for 90 sec in the autosampler fill loop (Rheodyne, 20µl volume) before being injected automatically (20µl) into the mobile phase prior to the column. To compensate for flaws in the Beckman System Gold software, the controlling Amstrad computer was informed that the autosampler had a 100µl fill loop and to inject 25µl (O'Dea, 1989).

Separation was carried out with a 4.6 x 250mm Beckman Ultrasphere ODS (C18) column (5µm silica particle diameter with 80 Angstrom pore size) protected by a 4.6 x 45mm guard column. Samples were separated on the column using gradient elution with the following mobile phase compositions. Mobile phase solution A contained 20% (v/v) 0.35M sodium propionate buffer pH 6.5 and 8% (v/v) acetonitrile (HPLC solvent, Fisons). The sodium propionate buffer was made up as follows: 49.69g disodium hydrogen phosphate (dibasic anhydrous, Sigma) was dissolved in pure water to which was added 18.7ml propionic acid (AnalaR, BDH), the pH was adjusted to 6.5 with 4M NaOH (AnalaR 160g l^{-1}, BDH) and the volume made up to 1 litre. Mobile phase solution B contained a higher organic solvent proportion and so was of

a less polar nature than solution A. Solution B contained 30% (v/v) acetonitrile (HPLC solvent, Fisons), 25% (v/v) methanol (HPLC solvent, Fisons) and 3% (v/v) dimethyl sulphoxide (Analytical reagent, Fisons).

The mobile phase composition was controlled by the separation method programme (System Gold software) which contained a pump time programming facility (Table 2.3.). The proportion of the less polar solution B was increased during each sample run (gradient elution) so enabling elution of the least polar amino acids within an acceptable time scale. The key amino acids aspartate, glutamate, asparagine, glutamine, and the homoserine internal standard, were eluted within the first 20min of this programme.

Table 2.3. Gradient elution programme for the separation of aspartate, glutamate, asparagine, glutamine and homoserine (model 116/126 pumps).

Time (min)	Flow rate (ml min^{-1})	Composition, solution A%	Composition, solution B%
0	2	98	2
15	2	80	20
20	2	80	20
25	2	0	100
45	2	0	100
47	2	98	2
50	2	98	2

Amino acids of a less polar nature than homoserine were flushed from the column unresolved by a subsequent 20min wash phase with 100% solution B. These unresolved amino acids were not analysed on a routine basis during the storage season. The entire complement of tuber primary free amino acids was analysed on

several occasions to provide a comparison with total free amino acid content determined by ninhydrin assay. On these occasions the following gradient elution programme was used (Table 2.4.).

Table 2.4. Gradient elution programme for the separation of all tuber primary free amino acids (model 116/126 pumps).

Time (min)	Flow rate (ml min^{-1})	Composition, solution A%	Composition, solution B%
0	2	98	2
6	2	88	12
19	2	88	12
23	2	85	15
25	2	85	15
32	2	50	50
46	2	50	50
56	2	0	100
70	2	0	100

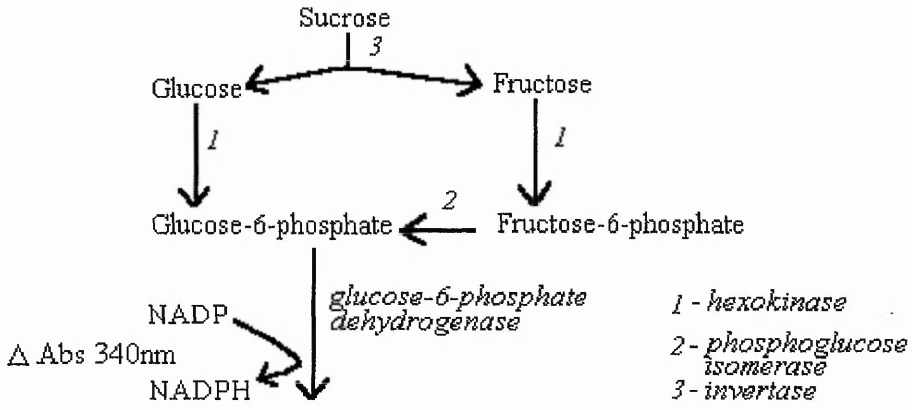
Amino acid peaks were detected by the model 157 fluorescence detector and the peak areas compared with that of the homoserine internal standard (2.5μM) when analysed by the software. When writing the separation method programmes, the system was calibrated for a range of amino acid concentrations and each amino acid assigned a retention time window. This allowed automated peak identification and quantification at the end of each sample run, the data and sample chromatograms being stored on the computer hard disc. Amino acid concentrations were obtained on a μM basis and converted to mg (amino acid) g^{-1}(freeze dried extract), final results were expressed as mg (amino acid) g^{-1}(tuber tissue fresh weight). The format of these calculations is detailed in the Appendix.

2.2.6. Determination of tuber tissue reducing sugar content.

Tuber tissue reducing sugar and sucrose content was determined by R.O.Williams and N.D.Turnbull using the enzymic analysis of freeze-dried tuber extracts. The enzymic sugar assay method used was a modified version (Williams and Cobb, 1992) of the microplate assay developed by Viola and Davies (1992).

Freeze-dried tuber extracts as used for amino acid and soluble protein determinations were resuspended in 5% (w/v) polyvinylpyrolidone (Sigma) and then diluted to within the concentration range of the calibration standards used. To each microplate sample well, 200µl of combined sugar standards or resuspended tuber extract were added. To these samples and calibration standards a sequential addition of enzymes was performed in order to determine the glucose, fructose and sucrose concentrations respectively. The sequence of enzyme addition was as follows: 30µl hexokinase (Technicon RA-Systems Reagents), 10µl phosphoglucose isomerase (Sigma) and 10µl invertase (Sigma). The sugar concentration at each stage was determined by measuring the change in absorbance at 340nm, caused by the reduction of NADP by glucose-6-phosphate dehydrogenase (Technicon RA-Systems Reagents), this enzyme being added at the start of the assay (Fig. 2.05.).

Each stage of the assay was allowed to reach completion (30-40 min) before the next enzyme was added, the absorbance change over each stage (measured on a plate reader) being proportional to the concentration of the appropriate sugar. Sugar standards in the concentration range of 5-50µg ml^{-1} were used to calibrate this absorbance change. Concentrations were expressed as mg g^{-1}(tuber tissue fresh weight) (see appendix for calculations).



1. Hexokinase
D-glucose or D-fructose + ATP → glucose / fructose-6-phosphate + ADP

2. Phosphoglucose isomerase
Fructose-6-phosphate → glucose-6-phosphate

3. Invertase
Sucrose + H_2O → D-glucose + D-fructose

Glucose-6-phosphate dehydrogenase
Glucose-6-phosphate + NADP → gluconate-6-phosphate + NADPH

Figure 2.05. Sequential enzymic assay of glucose, fructose and sucrose

2.2.7. Determination of fry colours.

Tuber fry colours were provided for each season by the Potato Marketing Board Experimental Station at Sutton Bridge in Lincolnshire. Tubers were sampled at regular intervals by Sutton Bridge staff from the same storage and temperature regimes as used in all other experiments. Pentland Dell and Record tubers were cut into French-fries and crisps respectively and fried at 180±5°C in vegetable oil for 3.5min (P.M.B. Sutton Bridge Experimental Station Annual Review, 1989). From each fried sample 15 French-fries or crisps were assessed for fry colour using the PMB scale. This assessment technique was based on the United States Department of Agriculture (U.S.D.A.) colour chart, fry colours being matched visually to those

displayed on the chart (0 = lightest and 7 = darkest). In addition, fry colours were also determined for the whole fried sample (25-30 French-fries or crisps) using an Agtron colorimeter. This device operates on the principle of light reflectance, the darker fry colour samples reflecting less light and hence scoring lower on the Agtron scale (90 = lightest and 0 = darkest), values of 18 units and above were deemed acceptable by processors.

2.3. RESULTS

2.3.1. The total free amino acid pool size of potato tuber tissue during prolonged tuber storage.

The total free amino acid pool size of tuber tissue as determined by ninhydrin assay, showed various trends in both Pentland Dell and Record tubers over the 4 storage seasons studied.

Table 2.5. The concentration range of total free amino acids in Pentland Dell and Record tubers stored at 5° and 10°C.

$\bar{x}$ (n=5) ± S.E. Expressed as mg g^{-1}(fresh weight)

Storage season	Pentland Dell 5°C	Pentland Dell 10°C
1989-90	10.42±0.52-18.97±2.52	7.73±1.18-23.20±4.15
1990-91	7.65±1.15-11.53±2.05	7.64±0.89-13.62±1.57
1991-92	10.45±1.05-15.61±1.21	5.66±0.79-20.81±3.76
1992-93	8.52±0.40-18.29±2.72	8.42±1.22-24.48±3.39
	Record 5°C	Record 10°C
1989-90	9.34±1.30-22.59±2.34	8.46±1.02-16.28±1.66
1990-91	5.69±1.25-16.90±3.28	6.24±1.08-23.37±5.84
1991-92	8.77±0.88-25.02±5.95	9.84±1.05-19.79±1.62
1992-93	8.00±1.90-22.99±2.47	8.40±0.78-14.57±1.85

The free amino acid pool of Pentland Dell fluctuated throughout storage in all 4 seasons studied, the degree of fluctuation being 1.5 to 3 fold greater at 10°C than 5°C (Table 2.5. and Figs. 2.06., 2.07., 2.08. and 2.09.). Two underlying trends of free amino acid content were discerned in Pentland Dell, firstly, a peak occurred at approximately 12 weeks in the 1989-90, 1991-92 and 1992-93 seasons, this happening at 10°C only in the 1991-92 and 1992-93 seasons (Figs. 2.06., 2.08. and 2.09.). Secondly, in all seasons with the exception of 1990-91, the pool size was observed to significantly increase ($p<0.05$) during mid to late storage (16-40 weeks). This rise was greatest in 1991-92 (49% at 5°C and 191% at 10°C) and 1992-93 (83% at 5°C and 161% at 10°C), in both these cases the upturn taking place between 17 and

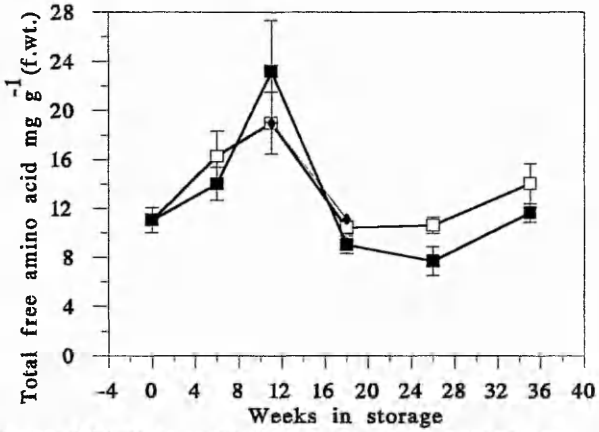


Figure 2.06. The total free amino acid content of Pentland Dell tubers stored at 5°C (□), 10°C (■) and reconditioned at 20°C (◆), 1989-90 storage season.

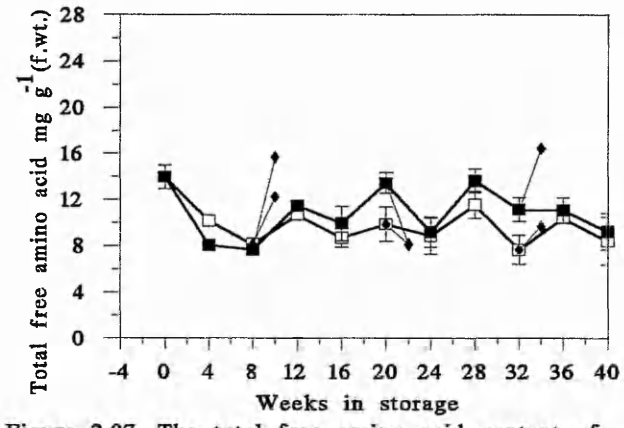


Figure 2.07. The total free amino acid content of Pentland Dell tubers stored at 5°C (□), 10°C (■) and reconditioned at 20°C (◆), 1990-91 storage season.

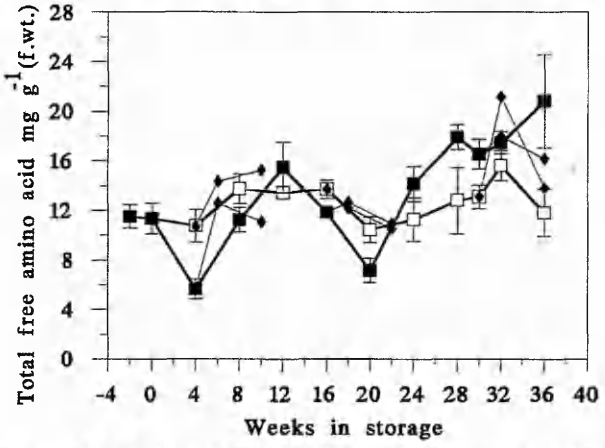


Figure 2.08. The total free amino acid content of Pentland Dell tubers stored at 5°C (□), 10°C (■) and reconditioned at 20°C (◆), 1991-92 storage season.

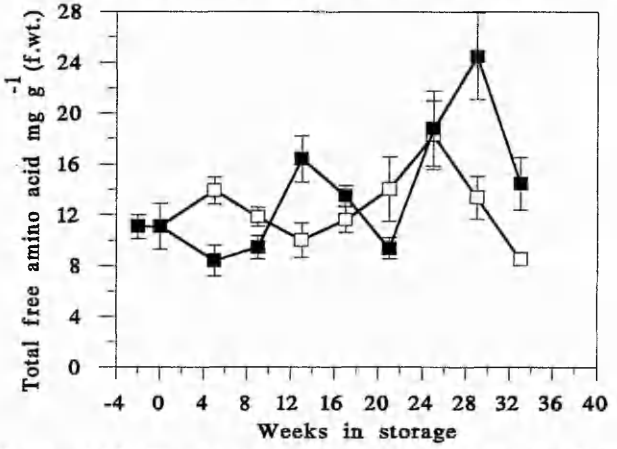


Figure 2.09. The total free amino acid content of Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1992-93 season.

20 weeks (Figs. 2.06., 2.07., 2.08. and 2.09.). In all but the 1989-90 season the increase in free amino acids appeared more pronounced at 10°C, this temperature effect being significant ($p<0.05$) in the 1991-92 and 1992-93 storage seasons.

Trends of free amino acid content with storage were similar in Record to Pentland Dell, an early season peak (4-11 weeks) being observed at 5°C in the 1989-90 and 1991-92 seasons (Figs. 2.10. and 2.12.). A significant ($p<0.05$) mid to late season upturn in free amino acid pool size took place in all seasons between 8 and 35 weeks, the majority of this rise taking place between 20 and 28 weeks in 1990-91, 1991-92 and 1992-93, and in 1989-90 between 26 and 35 weeks (Figs. 2.10., 2.11., 2.12. and 2.13.). The increase in free amino acids was 51, 24, 174 and 145% in 5°C stored tubers in the 1989-90, 1990-91, 1991-92 and 1992-93 seasons respectively, whereas at 10°C the rise was 50, 111, 101 and 20%. No consistent temperature effect was observed on the rise of free amino acid content in Record, with the exception of a 38% higher pool size at 10°C than 5°C after 28 weeks storage in 1990-91 (Table 2.5. and Fig. 2.11.). This temperature influence was significant at 95% confidence limits.

Reconditioning treatments did not produce a consistent effect on the total free amino acid pool size for either cultivar in any of the 4 seasons studied and in most cases no significant ($p<0.05$) reconditioning effects were observed (Figs. 2.06. to 2.13.). Exceptions to this trend included amino acid build up in 10°C stored Pentland Dell and Record as a result of both early and late term reconditioning in 1990-91 (Figs. 2.07. and 2.11.). A decrease as a result of late season reconditioning of 10°C stored Pentland Dell took place in 1991-92 (Fig. 2.08.). Also in this season, 5°C Record tubers demonstrated a drop in pool size as a result of early-term reconditioning for 2 weeks, however this trend was reversed over the final 4 weeks at 20°C resulting in an overall rise due to reconditioning (Fig. 2.12.).

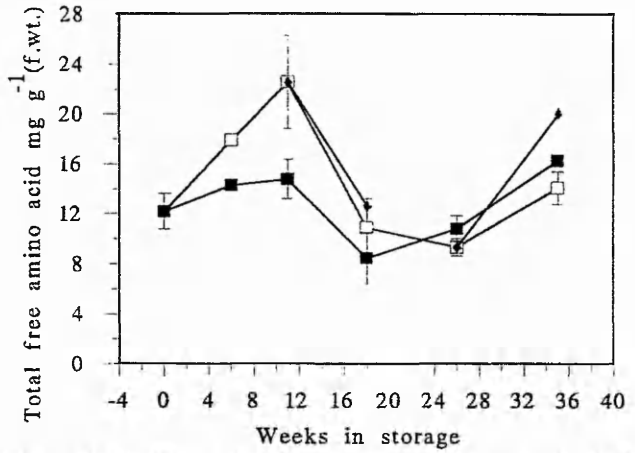


Figure 2.10. The total free amino acid content of Record tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1989-90 storage season.

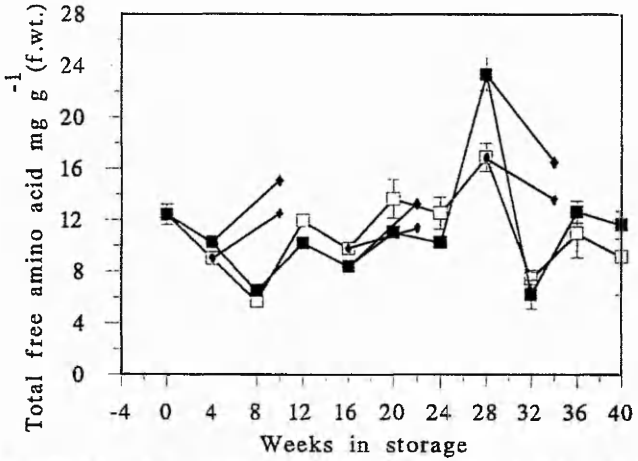


Figure 2.11. The total free amino acid content of Record tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1990-91 storage season.

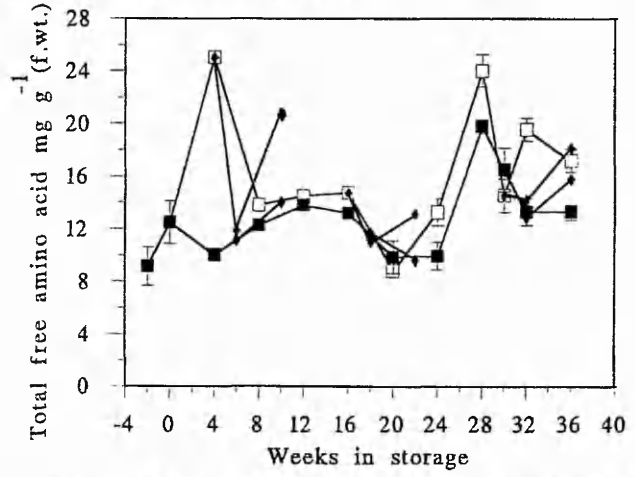


Figure 2.12. The total free amino acid content of Record tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1991-92 storage season.

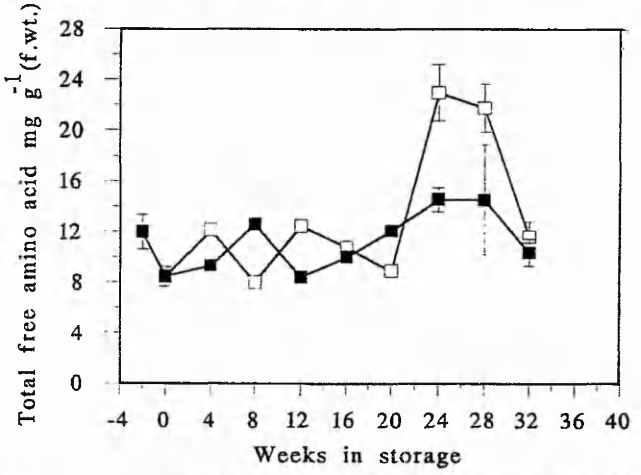


Figure 2.13. The total free amino acid content of Record tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

2.3.2. The composition of the tuber tissue free amino acid pool during prolonged storage.

An examination of the free amino acid pool by reverse-phase HPLC reveals a large proportion of the pool to consist of the amides asparagine and glutamine in both Pentland Dell and Record (Fig. 2.14.).

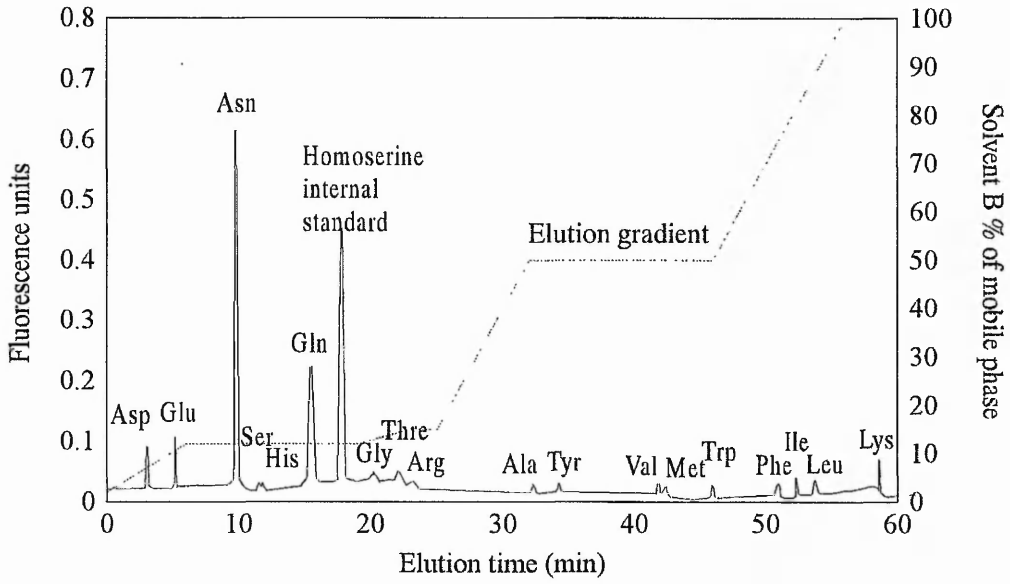


Figure 2.14. Typical composition of the tuber tissue free amino acid pool as separated by reverse-phase HPLC.

These storage amides exhibited several trends in pool size over the four storage seasons studied. No difference in the size of the amide pool as a result of tuber storage temperature was observed in every season in either cultivar.

The amide pool size in Pentland Dell was predominantly low over the first 16 weeks of each season, exceptions to this being the slight peak at 11 weeks in 1989-90 and a relatively high content on entry into storage in 1990-91 (Figs. 2.15., 2.16., 2.17. and 2.18.). A late season (16-36 weeks) rise in amide content was observed in Pentland Dell for all seasons examined (Figs. 2.15., 2.16., 2.17. and 2.18.), this increase was

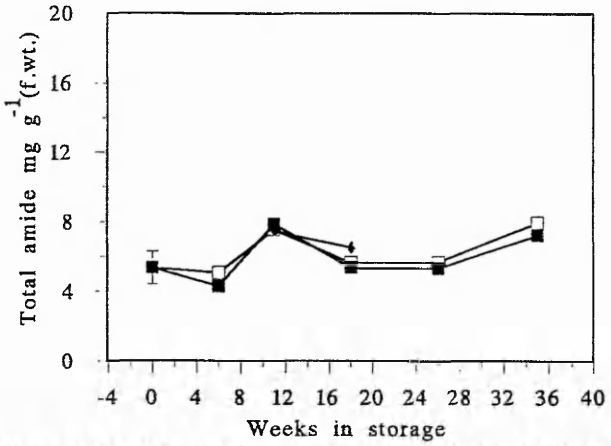


Figure 2.15. The total amide content of Pentland Dell tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1989-90 storage season.

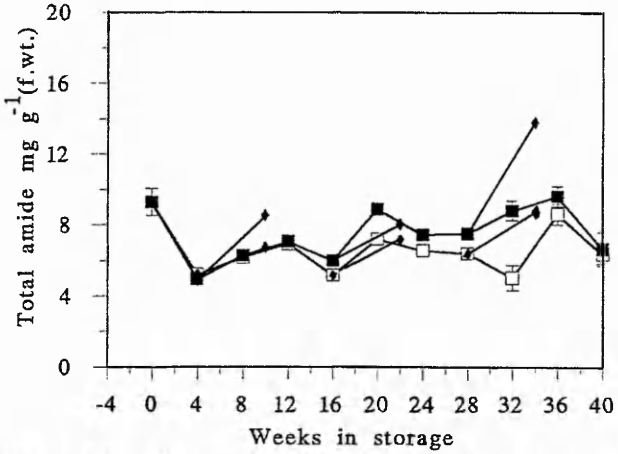


Figure 2.16. The total amide content of Pentland Dell tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1990-91 storage season.

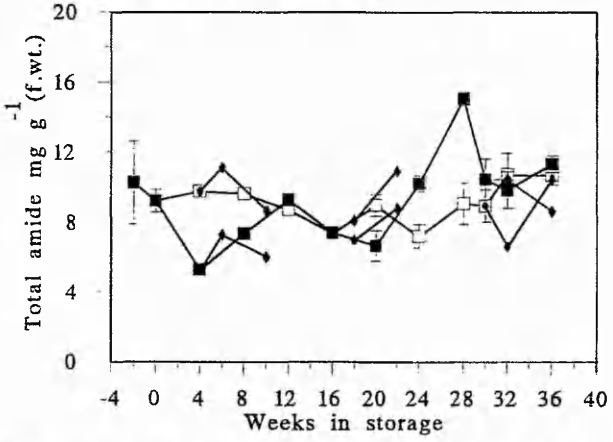


Figure 2.17. The total amide content of Pentland Dell tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1991-92 storage season.

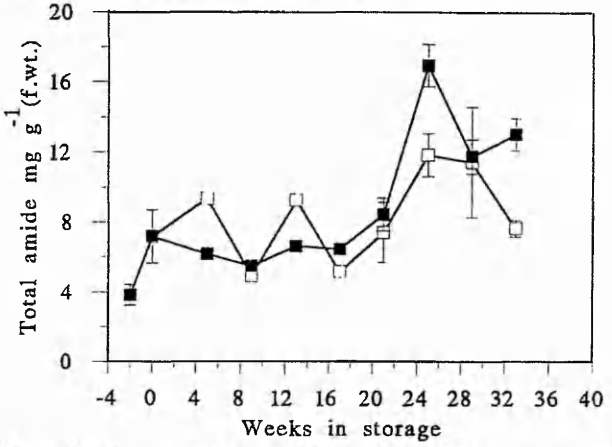


Figure 2.18. The total amide content of Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

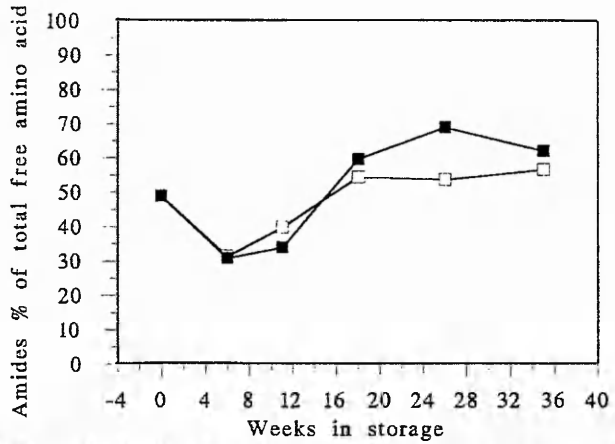


Figure 2.19. Total amide content as a percentage of the free amino acid pool in Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1989-90 storage season.

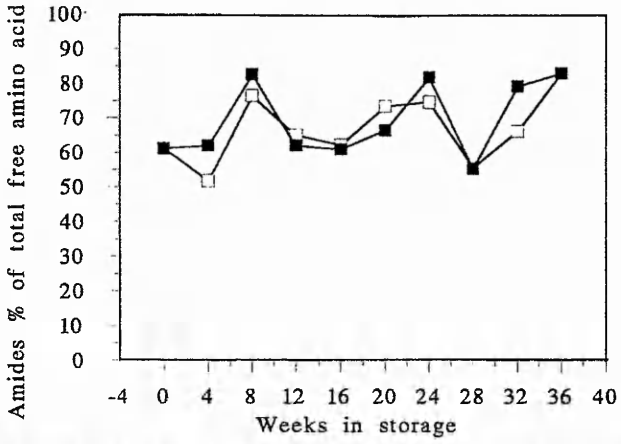


Figure 2.20. Total amide content as a percentage of the free amino acid pool in Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1990-91 storage season.

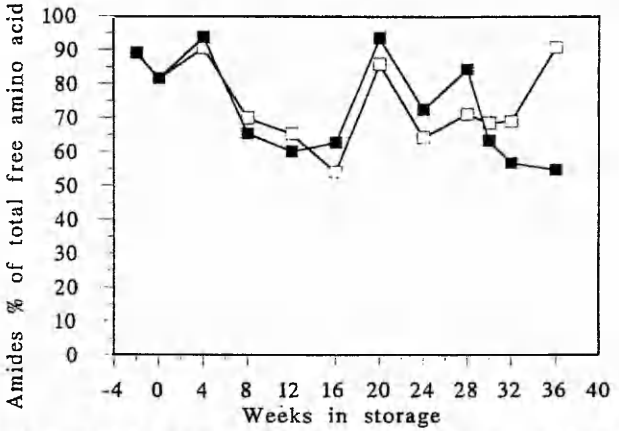


Figure 2.21. Total amide content as a percentage of the free amino acid pool in Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1991-92 storage season.

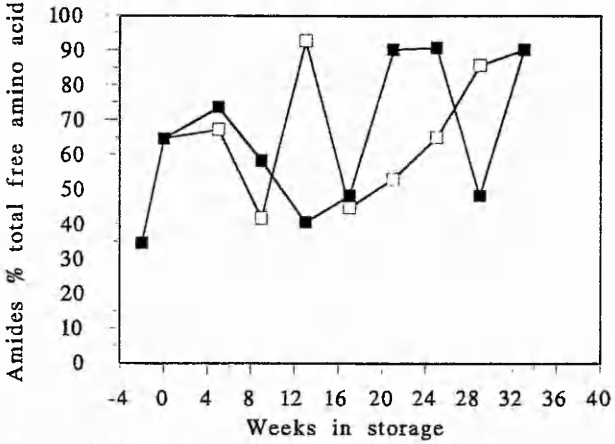


Figure 2.22. Total amide content as a percentage of the free amino acid pool in Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

significant ($p<0.05$) in 1991 at 10°C and 1992-93 at 5°C and 10°C. At 5°C this increase was 40%, 55%, 49% and 129% in the 1989-90, 1990-91, 1991-92 and 1992-93 seasons respectively, whereas at 10°C the upturn was 36%, 60%, 126% and 162%. The increase of amides was greater in tubers stored at 10°C than at 5°C by 12% in 1990-91, 66% in 1991-92 and 43% in 1992-93, the temperature influence was significant at 95% confidence limits in 1991-92 and 1992-93. In addition, this upturn of amide pool size generally corresponded with a growth in the proportion of the free amino acid pool comprising of the amides, this was most notable in 1992-93 (Figs. 2.19., 2.20., 2.21. and 2.22.). The extent to which amides made up the total free amino acid pool displayed no consistent temperature dependence, thus suggesting that any temperature differences in the amide pool were reflected by those in total free amino acid content.

Record also exhibited a relatively low amide content over most of the first 16-20 weeks of each season, however tubers demonstrated a comparatively high amide pool size on entry into storage in 1990-91 and 1991-92. In addition, other exceptions to the low early season amide content included peaks at 5°C in 1989-90 (6-11 weeks) and 1991-92 (4 weeks), and after 8 weeks storage at 10°C in 1992-93 (Figs. 2.23., 2.24., 2.25. and 2.26.). As in Pentland Dell, a late season (16-24 weeks) upturn of amide content was distinguished in Record in all seasons studied (Figs. 2.23., 2.24., 2.25. and 2.26.), although this was significant ($p<0.05$) at 5°C in only the 1991-92 and 1992-93 seasons and at 10°C in 1989-90, 1991-92 and 1992-93. In the 1989-90, 1990-91, 1991-92 and 1992-93 seasons this increase in 5°C stored tubers was 33%, 42%, 117% and 102% respectively, whereas in 10°C tubers the upturn was 46%, 95%, 123% and 147%. This rise was not always matched by an increase in the amide proportion of the free amino acid pool, a low proportion being displayed at 5°C and 10°C in 1991-92 and 5°C in 1992-93 (Figs. 2.27., 2.28., 2.29. and 2.30.). The late season growth of amide content exhibited a temperature dependence in the 1989-90,

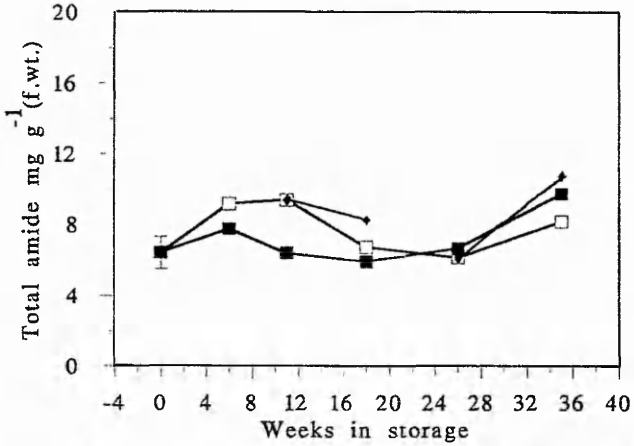


Figure 2.23. The total amide content of Record tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1989-90 storage season.

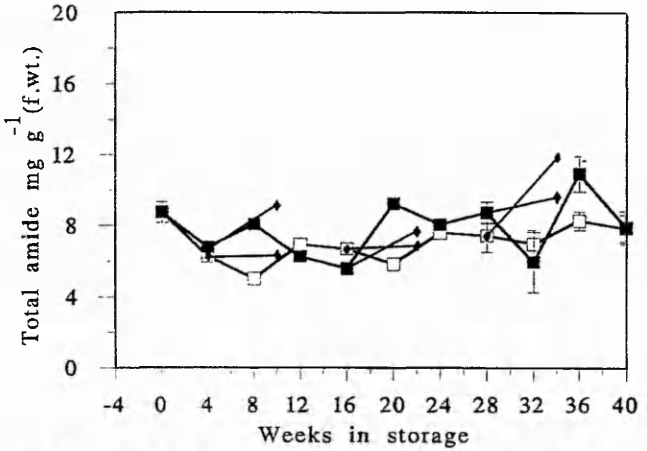


Figure 2.24. The total amide content of Record tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1990-91 storage season.

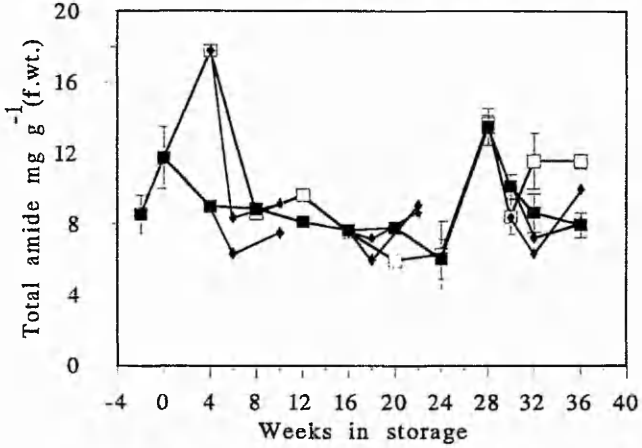


Figure 2.25. The total amide content of Record tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1991-92 storage season.

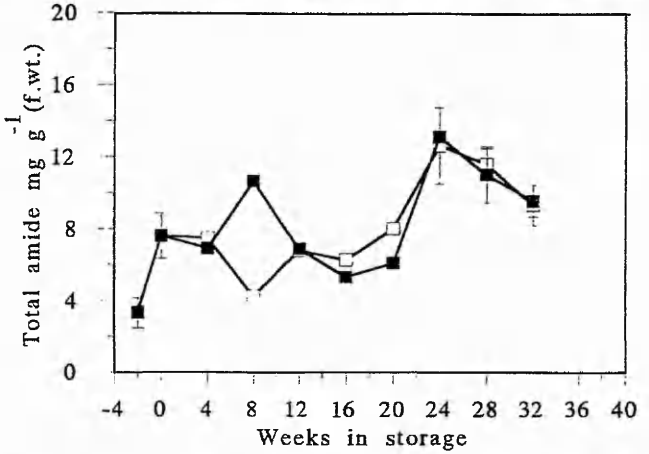


Figure 2.26. The total amide content of Record tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

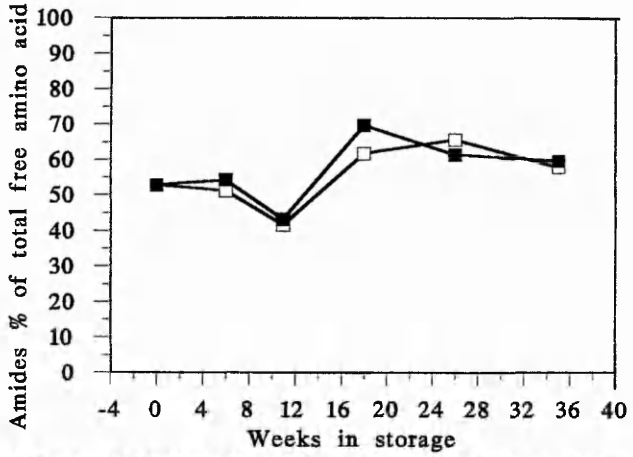


Figure 2.27. Total amide content as a percentage of the free amino acid pool in Record tubers stored at 5°C(□) and 10°C(■), 1989-90 storage season.

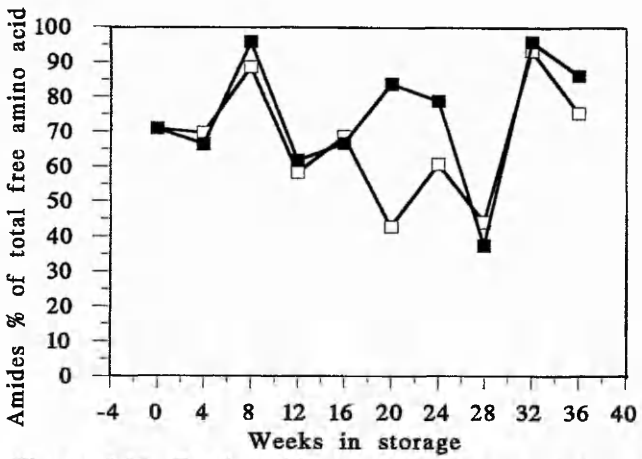


Figure 2.28. Total amide content as a percentage of the free amino acid pool in Record tubers stored at 5°C(□) and 10°C(■), 1990-91 storage season.

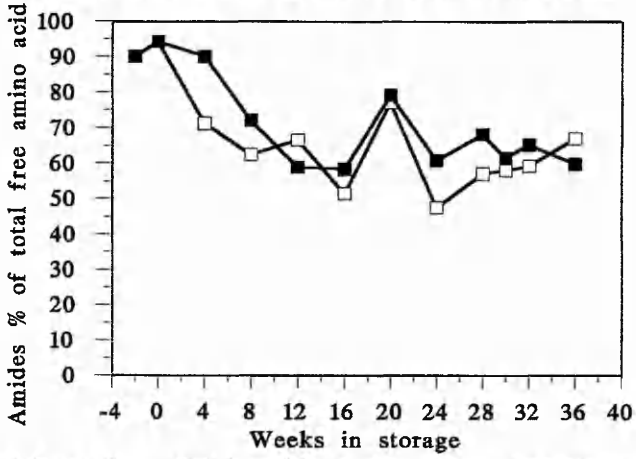


Figure 2.29. Total amide content as a percentage of the free amino acid pool in Record tubers stored at 5°C(□) and 10°C(■), 1991-92 storage season.

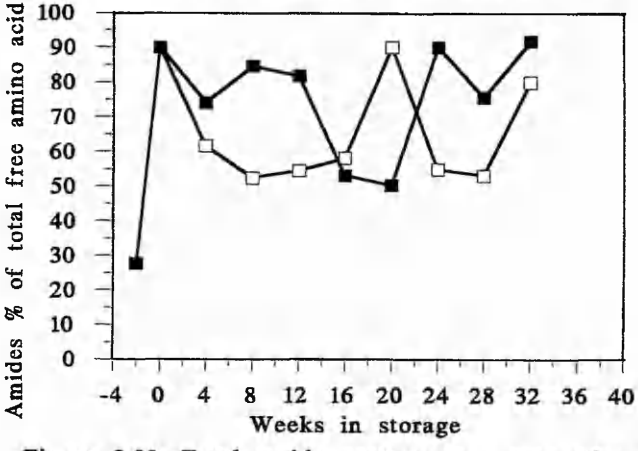


Figure 2.30. Total amide content as a proportion of the free amino acid pool in Record tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

1990-91 and 1992-93 seasons, however, this trend was not significant within 95% confidence limits and was not as prominent as in Pentland Dell. In all seasons studied, the amide content of Pentland Dell displayed greater fluctuation at 10°C than 5°C (Table 2.6.). This was not consistently observed in Record.

Reconditioning of tubers at 20°C for up to 6 weeks was generally noted to have no significant effect ($p>0.05$) on tuber amide content (Figs. 2.15., 2.16., 2.17., 2.23., 2.24. and 2.25.). In one exception, Pentland Dell tubers harvested in the autumn of 1990 and stored for 28 weeks at 10°C followed by 6 weeks reconditioning at 20°C, displayed a higher amide content ($p<0.05$) than tubers maintained at 10°C. In this instance, the amides increased to such an extent that they formed nearly the entire complement of tuber amino acids (Fig. 2.16.).

The relative proportions of asparagine and glutamine present in tuber tissue were observed to vary with season and cultivar. With the exception of 10°C stored tubers in 1989-90 ($p>0.05$), asparagine typically comprised a larger proportion (mg g^{-1}fresh weight basis) of the free amide pool in Pentland Dell than Record in each season ($p<0.05$), this difference being greatest in 1990-91 ($p<0.0005$) and 1992-93 ($p<0.005$) (Figs. 2.31. to 2.38.). Storage duration was also noted to effect these proportions in several instances. Pentland Dell in 1990-91 and 1991-92 displayed an invariable asparagine to glutamine ratio throughout storage, this also being observed for Record in 1989-90 and 1990-91 (Figs. 2.32., 2.33., 2.35. and 2.36.). Exceptions to this constant ratio included Pentland Dell in 1989-90, a reduction occurring in the proportion of the amides consisting of asparagine. This drop in the asparagine to glutamine ratio was brought about as glutamine increased to a greater extent than asparagine with storage duration (Fig. 2.31.). In 1992-93, both Pentland Dell and Record displayed an escalation in the proportion of asparagine, this being a result of a

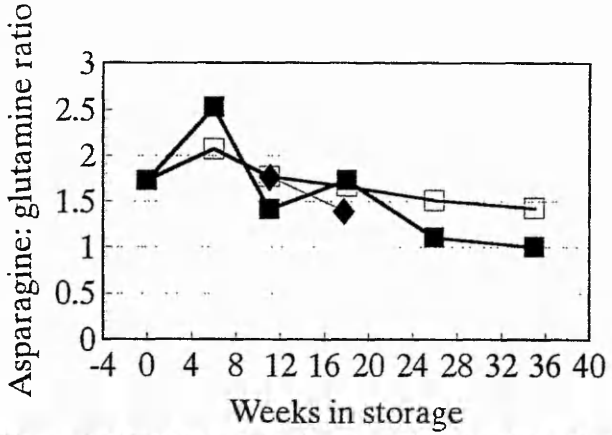


Figure 2.31. The ratio of asparagine to glutamine in Pentland Dell tubers (mg g fresh wt. basis) stored at 5°C(□), 10°C(■) and reconditioned at 20°C (◆),1989-90 storage season.

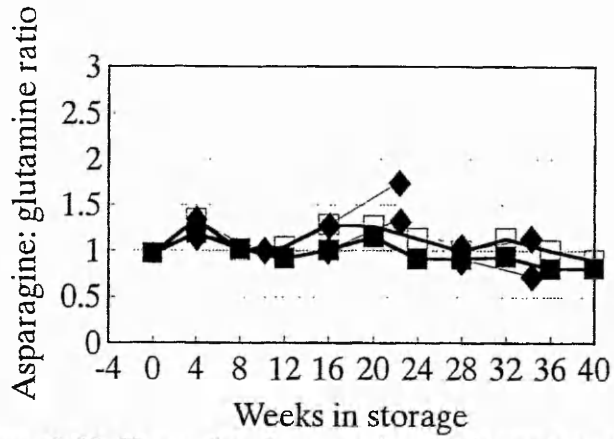


Figure 2.32. The ratio of asparagine to glutamine in Pentland Dell tubers (mg g fresh wt. basis) stored at 5°C(□), 10°C(■) and reconditioned at 20°C (◆),1990-91 storage season.

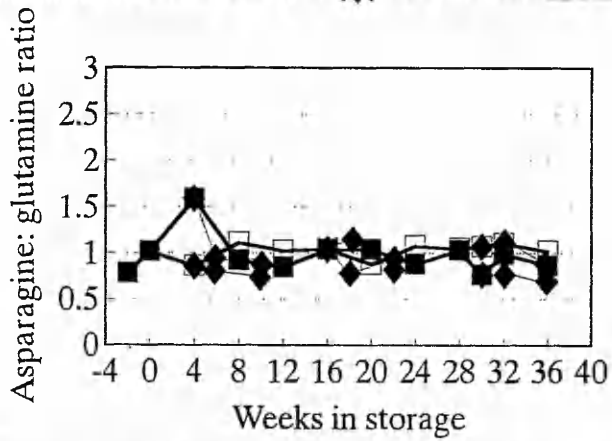


Figure 2.33. The ratio of asparagine to glutamine in Pentland Dell tubers (mg g fresh wt. basis) stored at 5°C(□), 10°C(■) and reconditioned at 20°C (◆),1991-92 storage season.

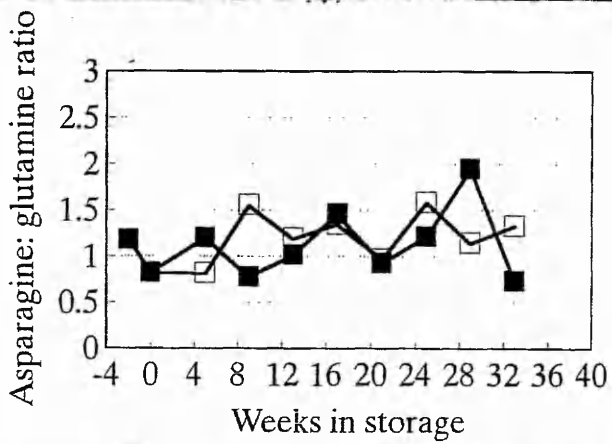


Figure 2.34. The ratio of asparagine to glutamine in Pentland Dell tubers (mg g fresh wt. basis) stored at 5°C(□) and 10°C(■), 1992-93 storage season.

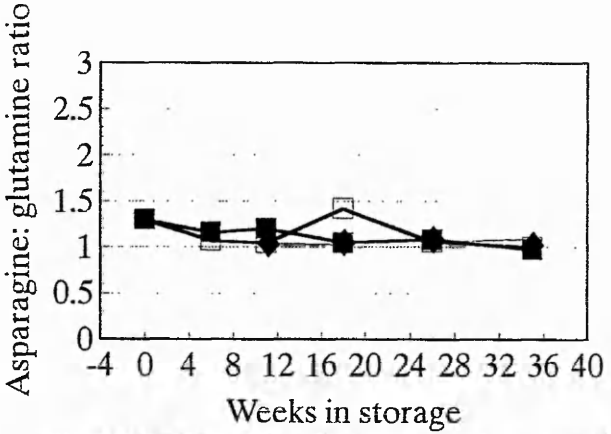


Figure 2.35. The ratio of asparagine to glutamine in Record tubers (mg g fresh wt. basis) stored at 5°C(□), 10°C(■) and reconditioned at 20°C (◆),1989-90 storage season.

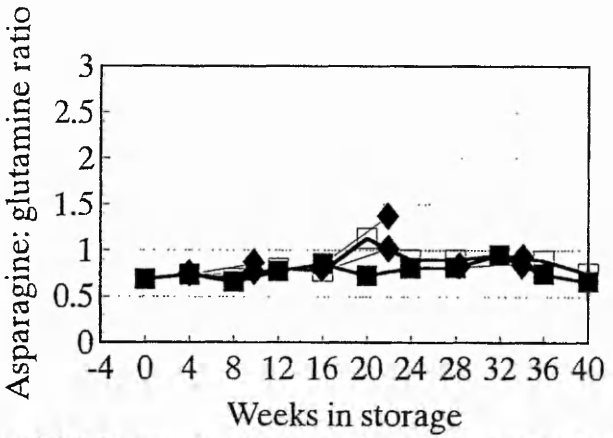


Figure 2.36. The ratio of asparagine to glutamine in Record tubers (mg g fresh wt. basis) stored at 5°C(□), 10°C(■) and reconditioned at 20°C (◆),1990-91 storage season.

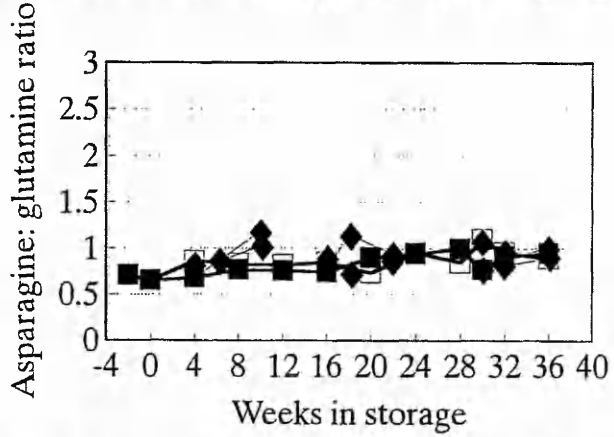


Figure 2.37. The ratio of asparagine to glutamine in Record tubers (mg g fresh wt. basis) stored at 5°C(□), 10°C(■) and reconditioned at 20°C (◆),1991-92 storage season.

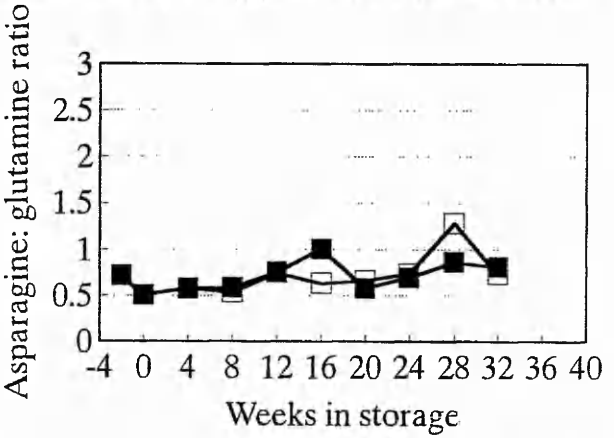


Figure 2.38. The ratio of asparagine to glutamine in Record tubers (mg g fresh wt. basis) stored at 5°C(□) and 10°C(■), 1992-93 storage season.

greater late season upturn of this amide relative to glutamine (Figs. 2.34. and 2.38.). In none of these cases was any effect of storage temperature observed ($p>0.05$), so indicating that the metabolism of these amides is strictly controlled at both 5°C and 10°C (Figs. 2.31. to 2.38.).

Reconditioning produced no consistent effect on the composition of the amide pool in all seasons, however both Pentland Dell and Record in 1990-91 exhibited a slight rise in this ratio brought about by mid-term reconditioning (from 16 weeks in storage) for 6 weeks at 20°C. This minor influence was due to a rise of asparagine (Figs. 2.32. and 2.36.). Early season reconditioning (from 4 weeks) for up to 6 weeks of Record tubers harvested in 1991 was also observed to enlarge this ratio in tubers previously stored at both 5°C and 10°C (Fig. 2.37.). This change was brought about by a greater decrease of glutamine relative to asparagine over this reconditioning period.

In addition to the amides, aspartate and glutamate formed the bulk of the remainder of the free amino acid pool (Fig. 2.14.), glutamate generally forming a larger proportion of the pool than aspartate (Table 2.6.). These amino acids were observed to account for less than 20% of the total free amino acid pool, and in many instances a far lower proportion.

Tuber glutamate content displayed near indentical trends throughout storage to those of the amide pool in all seasons studied (Fig. 2.39.) with the exception of the 1990-91 storage season. However, the concentration of glutamate was approximately 10% of that of the amide pool. As with asparagine and glutamine, glutamate exhibited a large late season rise in the 1991-92 and 1992-93 storage seasons for both Pentland Dell and Record tubers (Fig. 2.39.). For Pentland Dell in the 1991-92 and 1992-93 seasons respectively, the increase was 71% and 66% at 5°C, whereas at 10°C the accumulation was 103% and 318%. This upturn generally occurred between 20 and

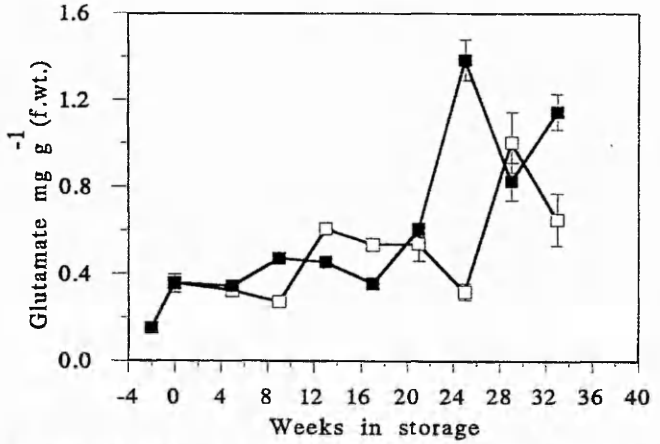


Figure 2.39. The glutamate content of Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

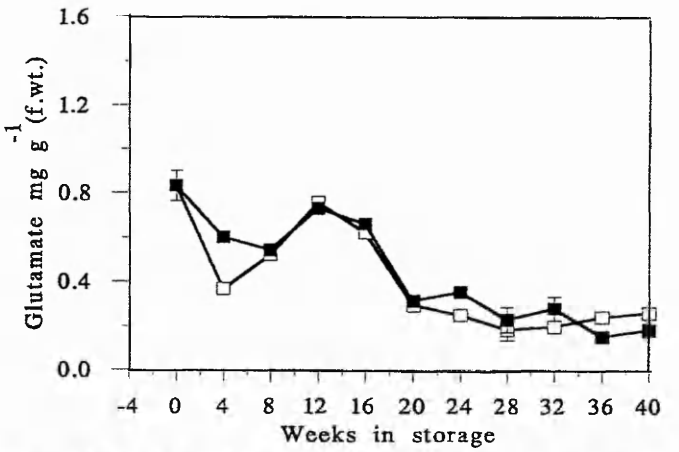


Figure 2.40. The glutamate content of Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1990-91 storage season.

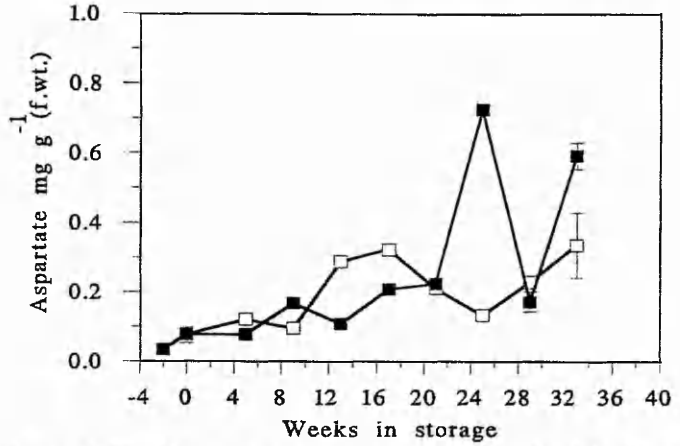


Figure 2.41. The aspartate content of Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

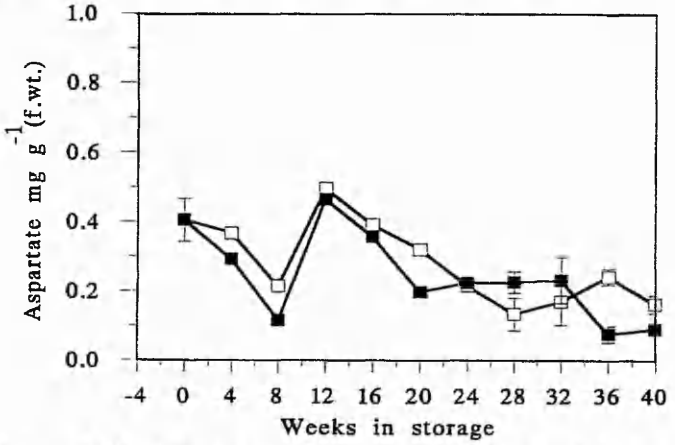


Figure 2.42. The aspartate content of Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1990-91 storage season.

32 weeks in storage, and for Pentland Dell tubers in the 1991-92 and 1992-93 seasons was greatest ($p<0.05$) in those tubers stored at 10°C (Fig. 2.39.). During the 1990-91 storage season, the glutamate content of both Pentland Dell and Record tubers was observed to decline by over 60% over the first 20 weeks of storage (Fig. 2.40.), the pool then remaining at a low concentration until the end of storage. This decline was independent of storage temperature (Fig. 2.40.). As a seasonal trend, glutamate decreased as a proportion of the total free amino acid pool.

Table 2.6. The concentration ranges of major amino acids during storage.

$\bar{x}$ (n=5) ± S.E. Concentrations expressed as mg g^{-1}(fresh weight)

Cultivar and season	Aspartate	Glutamate	Amides (Asn+Gln)
Pentland Dell 5°C			
1989-90	0.06±0.02-0.43±0.13	0.40±0.03-1.04±0.06	5.09±0.57-7.96±0.41
1990-91	0.13±0.04-0.50±0.02	0.19±0.03-0.83±0.07	5.05±0.07-8.62±1.01
1991-92	0.14±0.08-0.90±0.07	0.65±0.12-1.16±0.23	6.65±1.25-10.75±1.52
1992-93	0.03±0.01-0.34±0.20	0.15±0.03-1.00±0.15	3.82±0.60-11.86±2.09
Pentland Dell 10°C			
1989-90	0.04±0.01-0.35±0.09	0.42±0.05-0.96±0.08	4.32±0.58-7.88±0.64
1990-91	0.08±0.03-0.46±0.02	0.15±0.03-0.83±0.07	4.97±0.54-9.65±1.46
1991-92	0.04±0.02-0.78±0.11	0.65±0.12-1.32±0.14	5.30±0.88-15.07±2.17
1992-93	0.03±0.01-0.59±0.16	0.15±0.02-1.14±0.24	3.82±0.60-16.98±2.72
Record 5°C			
1989-90	0.08±0.02-0.31±0.10	0.48±0.04-0.80±0.17	6.13±0.73-9.38±0.93
1990-91	0.00±0.00-0.24±0.01	0.08±0.00-0.67±0.08	5.02±0.80-8.77±0.59
1991-92	0.06±0.03-0.89±0.14	0.55±0.11-1.39±0.07	5.93±0.41-17.77±1.90
1992-93	0.01±0.00-0.78±0.05	0.08±0.02-0.94±0.08	3.31±0.84-12.61±3.89
Record 10°C			
1989-90	0.08±0.03-0.28±0.13	0.51±0.05-0.77±0.10	5.89±0.60-9.71±0.96
1990-91	0.03±0.03-0.32±0.04	0.13±0.00-0.67±0.08	5.60±0.93-10.92±1.62
1991-92	0.08±0.07-0.53±0.09	0.40±0.06-1.13±0.14	6.04±0.82-13.48±1.65
1992-93	0.01±0.00-0.42±0.04	0.08±0.02-1.00±0.07	3.31±0.84-13.12±2.01

As for glutamate, aspartate exhibited similar trends in pool size to those of the amides in all seasons studied (Fig. 2.41.), with the exception of the 1990-91 storage season. In the 1991-92 and 1992-93 seasons a large late season rise of aspartate was observed for both Pentland Dell and Record ($p<0.05$). In Pentland Dell stored at 5°C the magnitude of this increase in 1991-92 and 1992-93 was 261% and 253%, whereas at 10°C the rise was 314% and 577% respectively (Fig. 2.41.). Apart from the above upturn of aspartate content in Pentland Dell in 1992-93, no clear effects of storage temperature were observed for other seasons or for Record tubers. During the 1990-91 storage season, aspartate decreased by 50% over the first 24 weeks of storage at both 5° and 10°C, the pool size subsequently remaining constant until the end of storage (Fig. 2.42.). Aspartate also showed an overall decrease as a proportion of the total free amino acid pool in the 1990-91 storage season. Reconditioning of tubers at 20°C for up to 6 weeks at early, mid and late intervals in storage was observed to have no significant effect ($p>0.05$) upon either aspartate or glutamate content.

All other amino acids apart from aspartate, glutamate, asparagine and glutamine generally comprised less than 20% of the total free amino acid pool (mg g^{-1} fresh weight basis). In many instances this proportion was far lower, this being most notable over the latter half of the 1990-91, 1991-92 and 1992-93 storage seasons for both Pentland Dell and Record tubers.

2.3.3. Amino acids as determinants of fry colour intensity.

The total free amino acid content of both Pentland Dell and Record, was invariably observed to be in excess with respect to the total reducing sugar pool size in all seasons studied (Table 2.7.). At any one point in time, tubers stored at 5°C were generally observed to contain higher total reducing sugar concentrations than those stored at 10°C (Table 2.7.). Exceptions to this trend occurred within the first 8 weeks

of storage for Pentland Dell in 1992-93 and Record in 1989-90 and 1990-91 (Figs. 2.46.a., 2.47.a. and 2.48.a.).

Table 2.7. The concentration range of total reducing sugars and free amino acids during prolonged storage.

$\bar{x}$ (n=5) ± S.E. Concentrations expressed as mg g^{-1}(fresh weight)

Pentland Dell 5°C Storage season	Total reducing sugars	Total free amino acids
1989-90	1.84±0.39-3.35±0.66	10.42±0.52-18.97±2.52
1990-91	0.88±0.36-6.75±0.28	7.65±1.15-11.53±2.05
1991-92	0.85±0.13-5.95±0.37	10.45±1.05-15.61±1.21
1992-93	0.81±0.18-9.81±1.36	8.52±0.40-18.29±2.72
Pentland Dell 10°C Storage season		
1989-90	0.01±0.01-1.26±0.43	7.73±1.18-23.20±4.15
1990-91	0.11±0.10-5.59±0.88	7.64±0.89-13.62±1.57
1991-92	0.85±0.13-3.31±1.10	5.66±0.79-20.81±3.76
1992-93	0.81±0.18-3.17±0.61	8.42±1.22-24.48±3.39
Record 5°C Storage season		
1989-90	0.45±0.16-4.71±1.06	9.34±1.30-22.59±2.34
1990-91	0.52±0.13-4.16±0.56	5.69±1.25-16.90±3.28
1991-92	1.51±0.25-9.00±1.37	8.77±0.88-25.02±5.94
1992-93	0.66±0.08-7.13±1.21	8.00±1.90-21.78±2.39
Record 10°C Storage season		
1989-90	0.34±0.01-2.14±0.01	8.46±1.02-16.28±1.66
1990-91	0.33±0.23-3.81±0.76	6.24±1.08-23.37±5.84
1991-92	0.40±0.12-3.78±0.84	9.84±1.05-19.79±1.62
1992-93	0.29±0.07-1.25±0.39	8.40±1.07-14.57±1.85

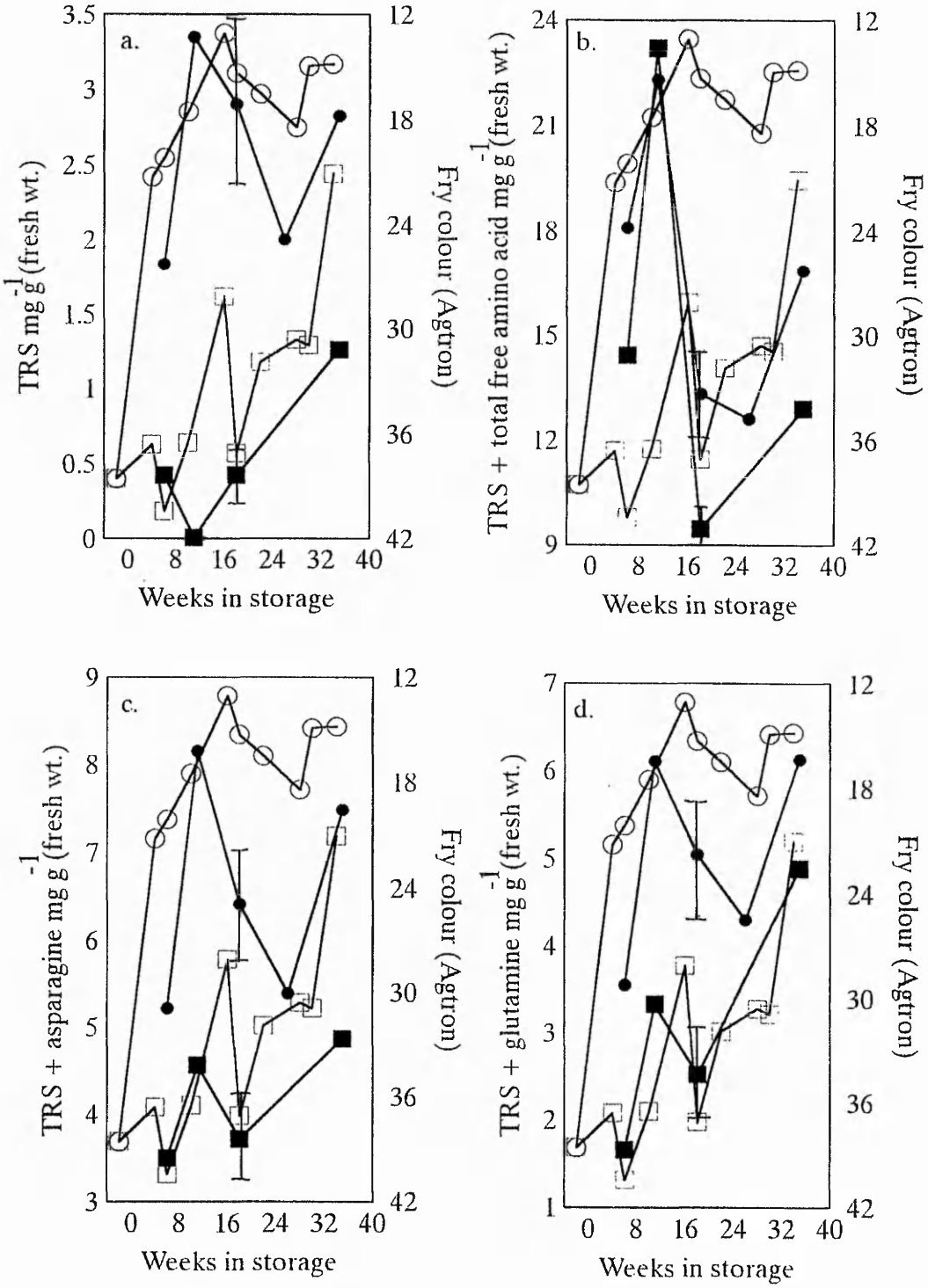
The larger reducing sugar pool of both Pentland Dell and Record stored at 5°C was reflected in the majority of instances by a darker fry colour than for those tubers stored at 10°C for the same duration. A distinct relationship of reducing sugar content to fry colour was hence evident (Figs. 2.43.a., 2.44.a., 2.45.a., 2.47.a., 2.48.a., 2.49.a., 2.50.a. and 2.51.a.). An exception to this trend was noted in Pentland Dell stored at 10°C in 1990-91, 1991-92 and 1992-93 (Figs. 2.44., 2.45. and 2.47.). These tubers demonstrated a pronounced deterioration of fry colour during mid to late

storage (from 6 weeks onwards in 1990-91 and 22 weeks in 1991-92 and 1992-93). In these instances, the degeneration of fry colour did not correspond to a rise in reducing sugar content, the pool not increasing until after 32 weeks in 1990-91 and 1991-92, and not at all in 1992-93 (Figs. 2.44.a., 2.45.a. and 2.47.a.). The sum of reducing sugars and amino acids displayed a marked increase from 8 weeks in 1990-91 and 20 weeks in 1991-92 and 1992-93, this trend being in step with the worsening fry colour (Figs. 2.44.b., 2.45.b. and 2.47.b.). Regression analysis of the relationship between reducing sugar content and fry colour throughout storage demonstrated a relatively weak correlation in Pentland Dell stored at 10°C in the above seasons (Table 2.8.). The inclusion of total free amino acids in this relationship improved the correlation in these instances, suggesting an influence of the free amino acid pool in fry colour production (Fig. 2.46. and Table 2.8.).

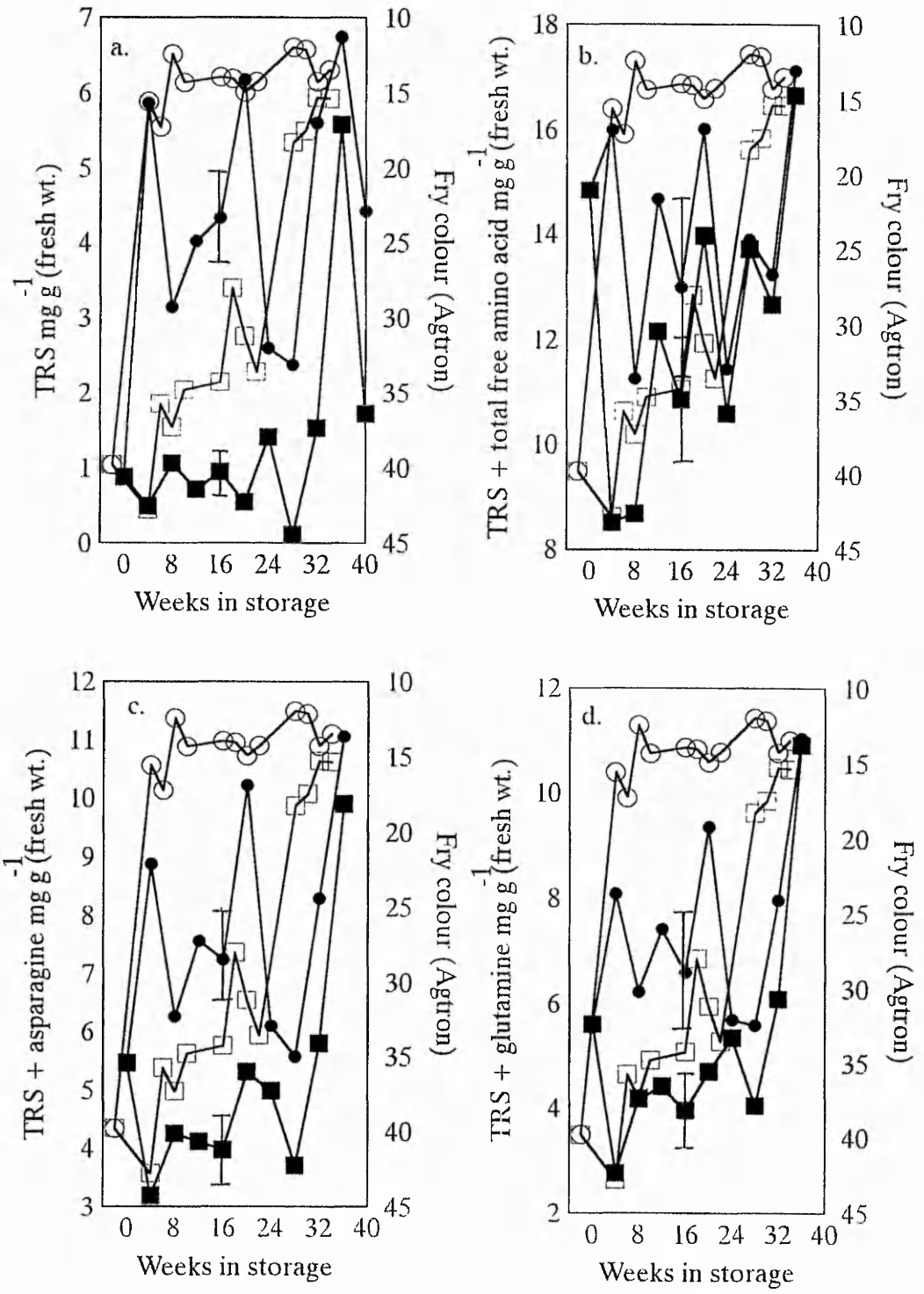
The individual amides also displayed influence on fry colour production for 10°C stored Pentland Dell in the 1990-91, 1991-92 and 1992-93 seasons. The mid to late season upturn of both asparagine and glutamine coincided with the deterioration in fry colour (Figs.2.44., 2.45. and 2.47.), the degree of this effect on fry colour intensity relative to that of the total free amino acid pool varying between seasons (Table 2.8.).

In tubers of Pentland Dell stored at 5°C, reducing sugars alone were the most reliable measure of potential fry colour quality in all seasons, the high pool size resulting in poor fry colours (Figs. 2.43., 2.44., 2.45., 2.47. and Table 2.8.). The sum of reducing sugar and amino acid or amide content did not improve upon the above correlation of fry colour with reducing sugars alone (Figs. 2.43., 2.44., 2.45., 2.47. and Table 2.8.).

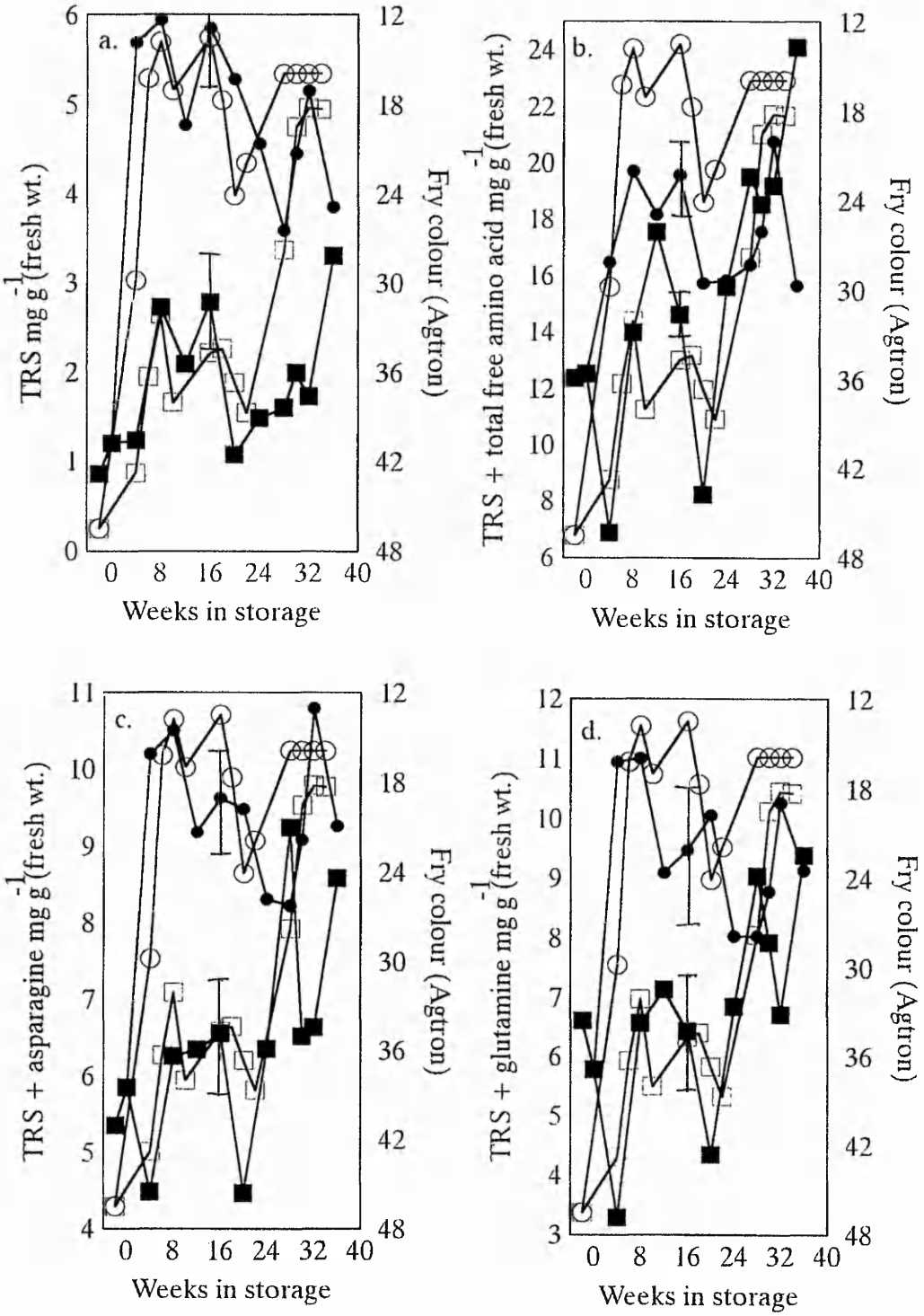
Neither the total free amino acid or individual amide contents of Record displayed a clear-cut influence upon fry colour production at either storage temperature (Figs. 2.48., 2.49., 2.50. and 2.51.). Unlike in Pentland Dell, the mid to late season upturn of



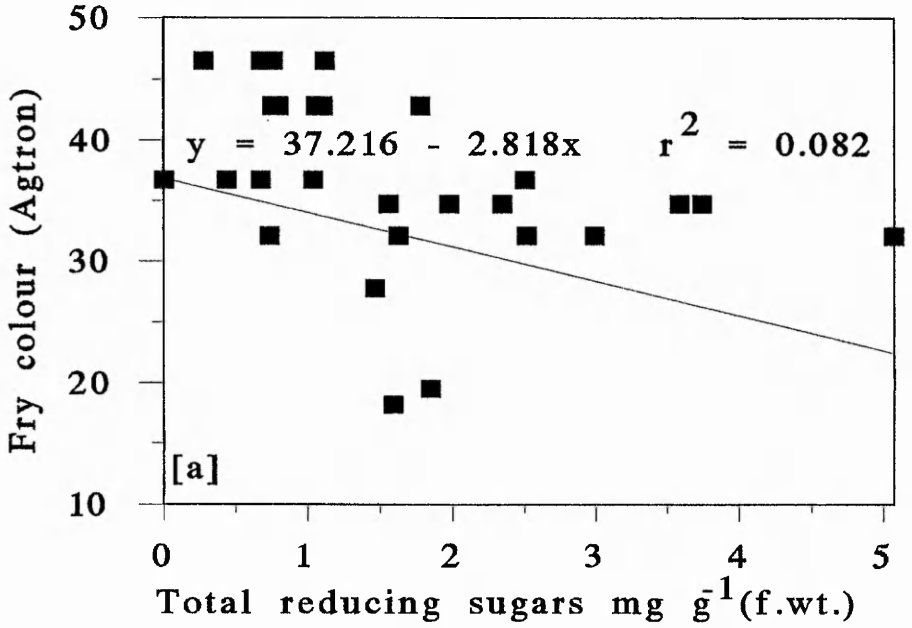
Figures 2.43.a, b, c and d. Fry colour by Agtron 5° (○) and 10°C (□). [a] compared to reducing sugar content, [b] the sum of reducing sugar and amino acid content, [c] the sum of reducing sugars and asparagine and [d] the sum of reducing sugars and glutamine in 5° (●) and 10°C (■) stored Pentland Dell tubers, 1989-90 storage season.

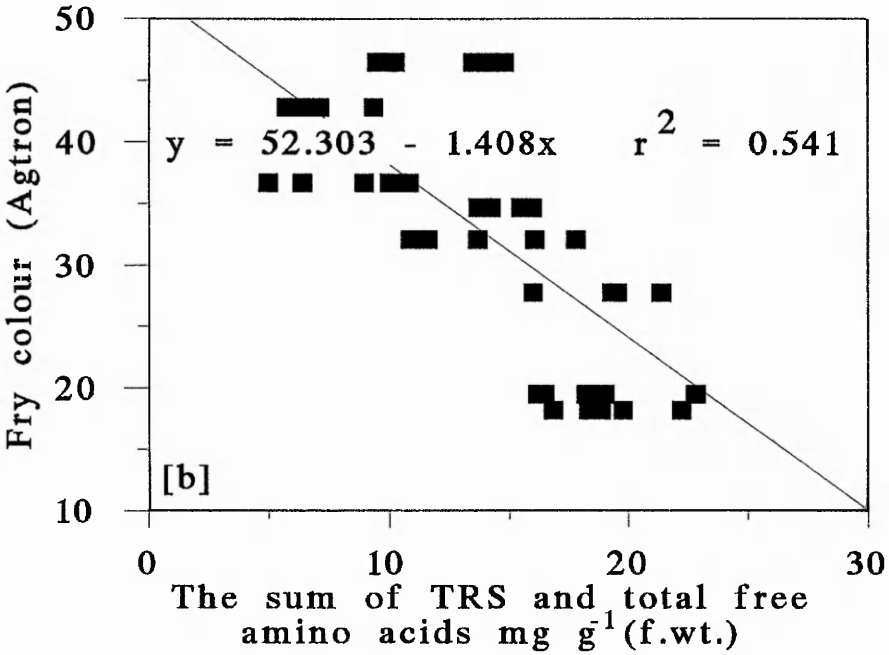


Figures 2.44.a, b, c and d. Fry colour by Agtron 5° (○) and 10°C (□). [a] compared to reducing sugar content, [b] the sum of reducing sugar and amino acid content, [c] the sum of reducing sugars and asparagine and [d] the sum of reducing sugars and glutamine in 5° (●) and 10°C (■) stored Pentland Dell tubers, 1990-91 storage season.

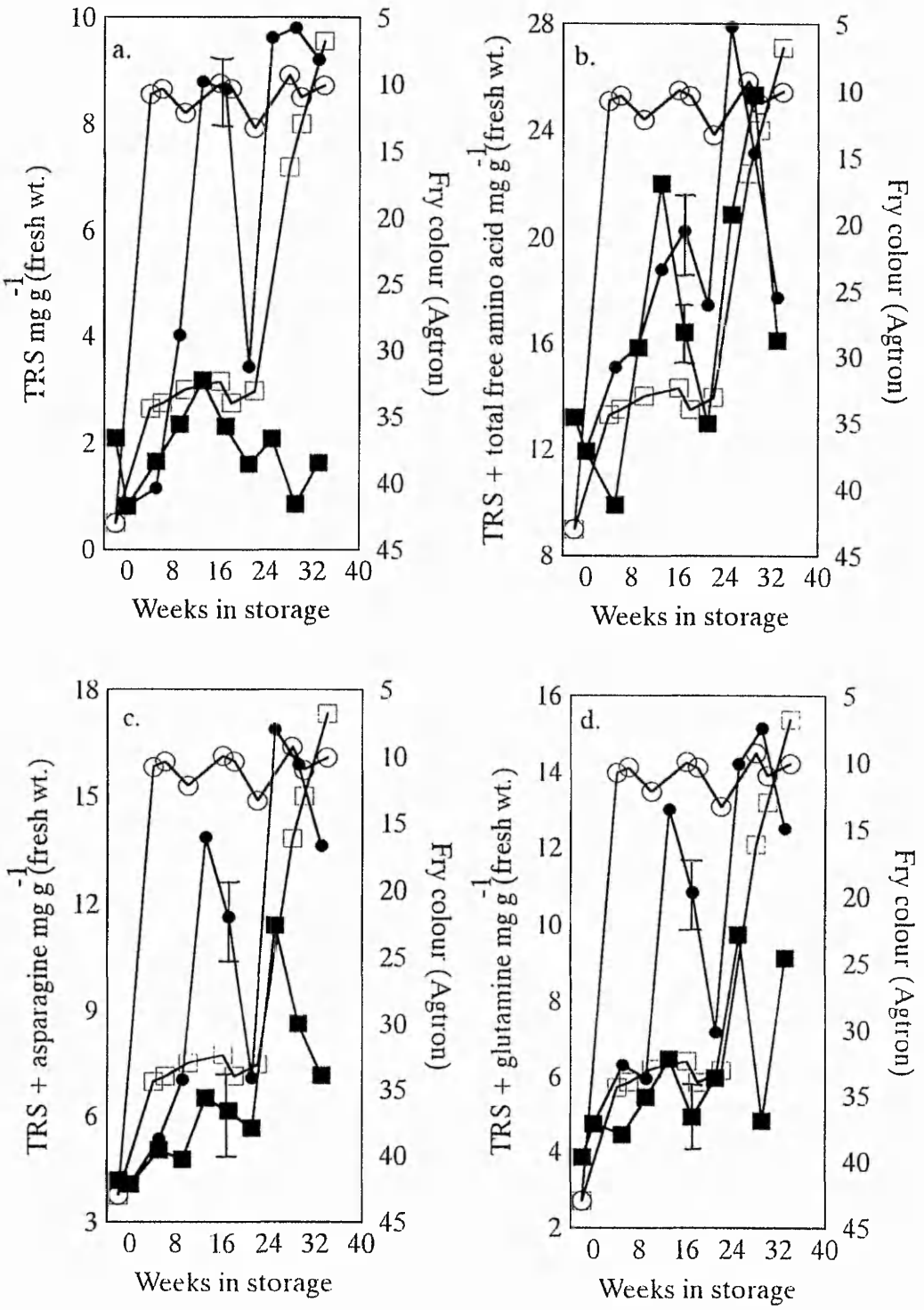


Figures 2.45.a, b, c and d. Fry colour by Agtron 5° (○) and 10°C (□), [a] compared to reducing sugar content, [b] the sum of reducing sugar and amino acid content, [c] the sum of reducing sugars and asparagine and [d] the sum of reducing sugars and glutamine in 5° (●) and 10°C (■) stored Pentland Dell tubers, 1991-92 storage season.

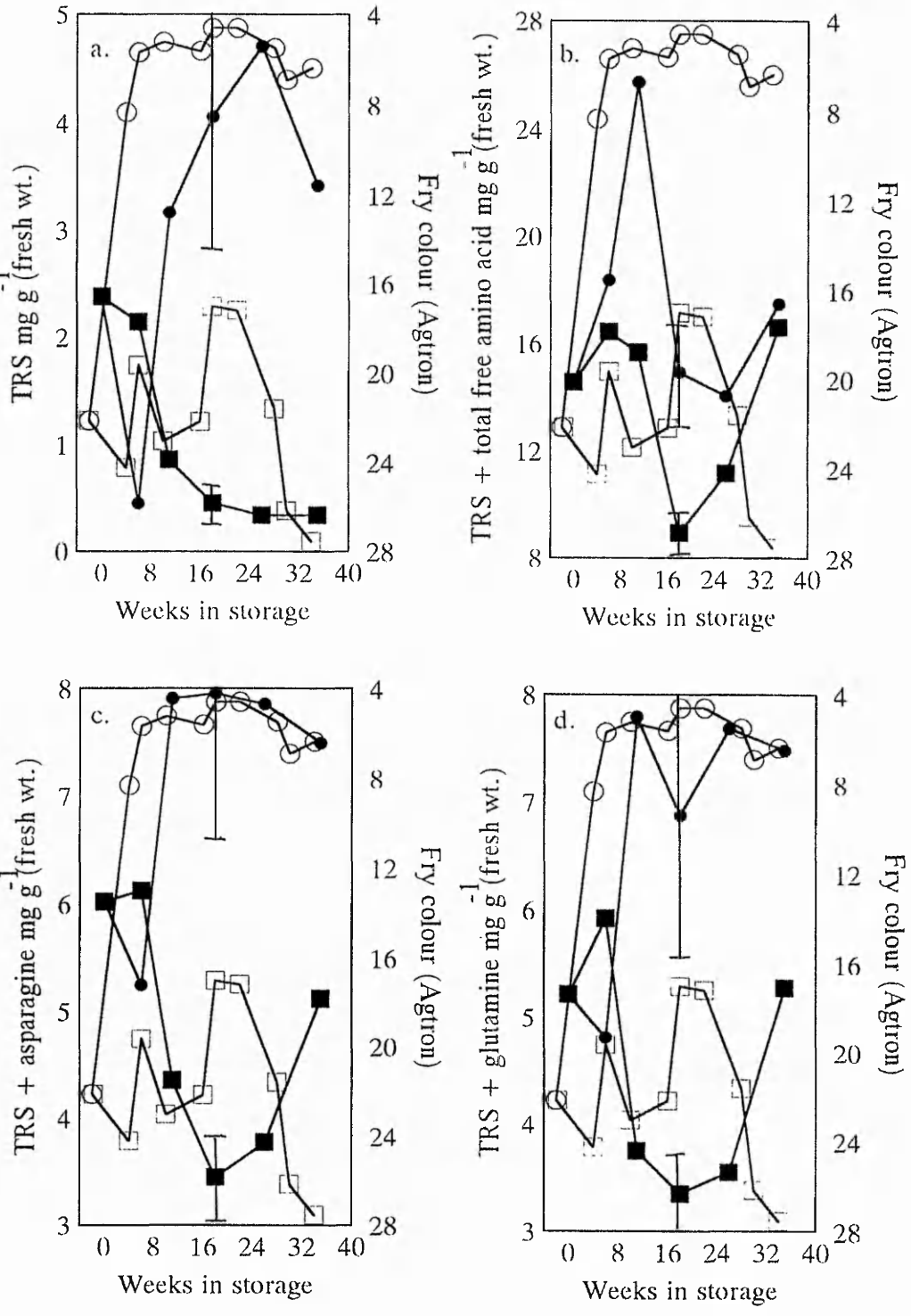




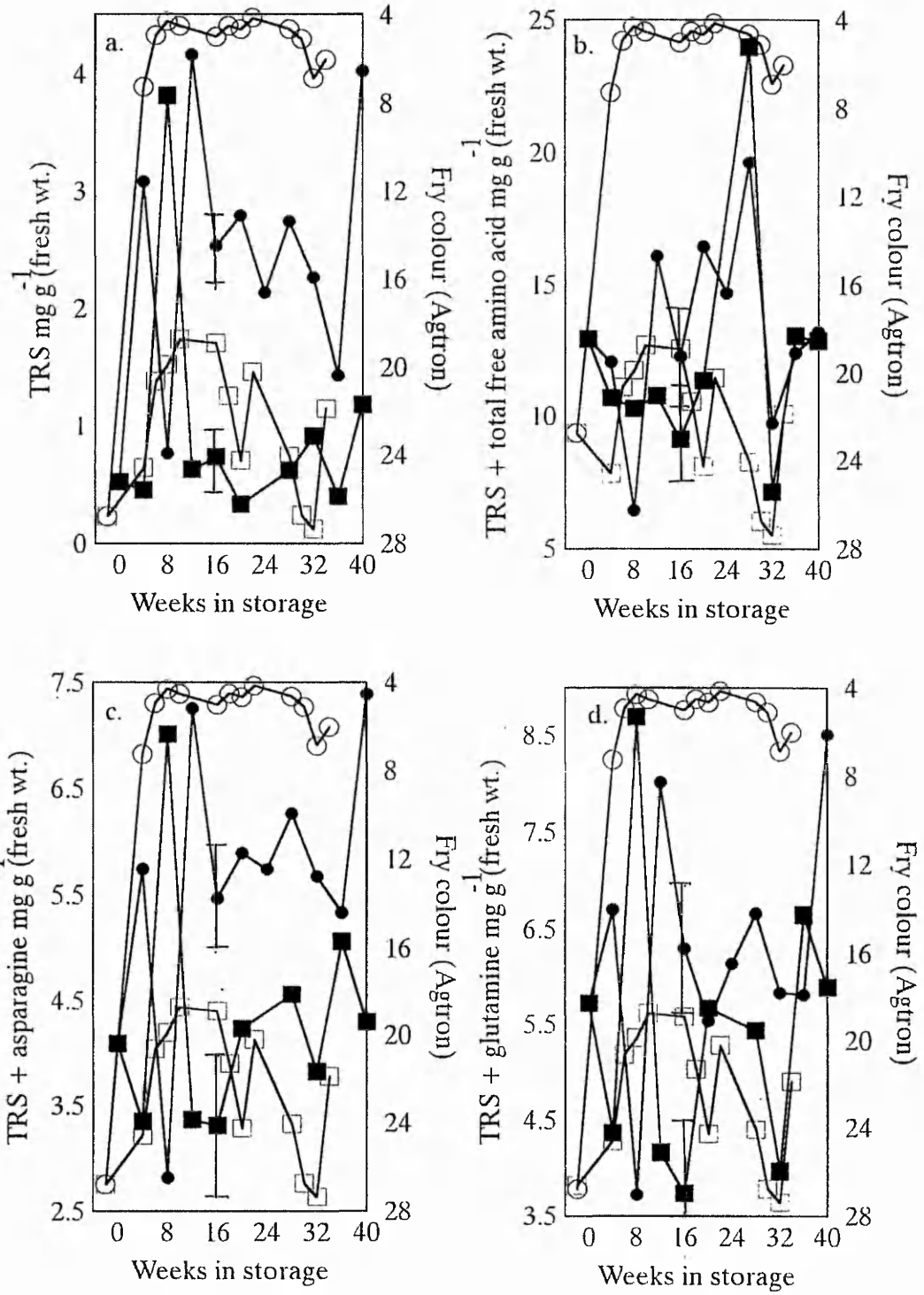
Figures 2.46. a. and b. The relationship of fry colour with: [a] total reducing sugars and [b] the sum of total reducing sugars and free amino acids (mg g^{-1}fresh wt.) for Pentland Dell tubers stored at 10°C, 1991-92 storage season.



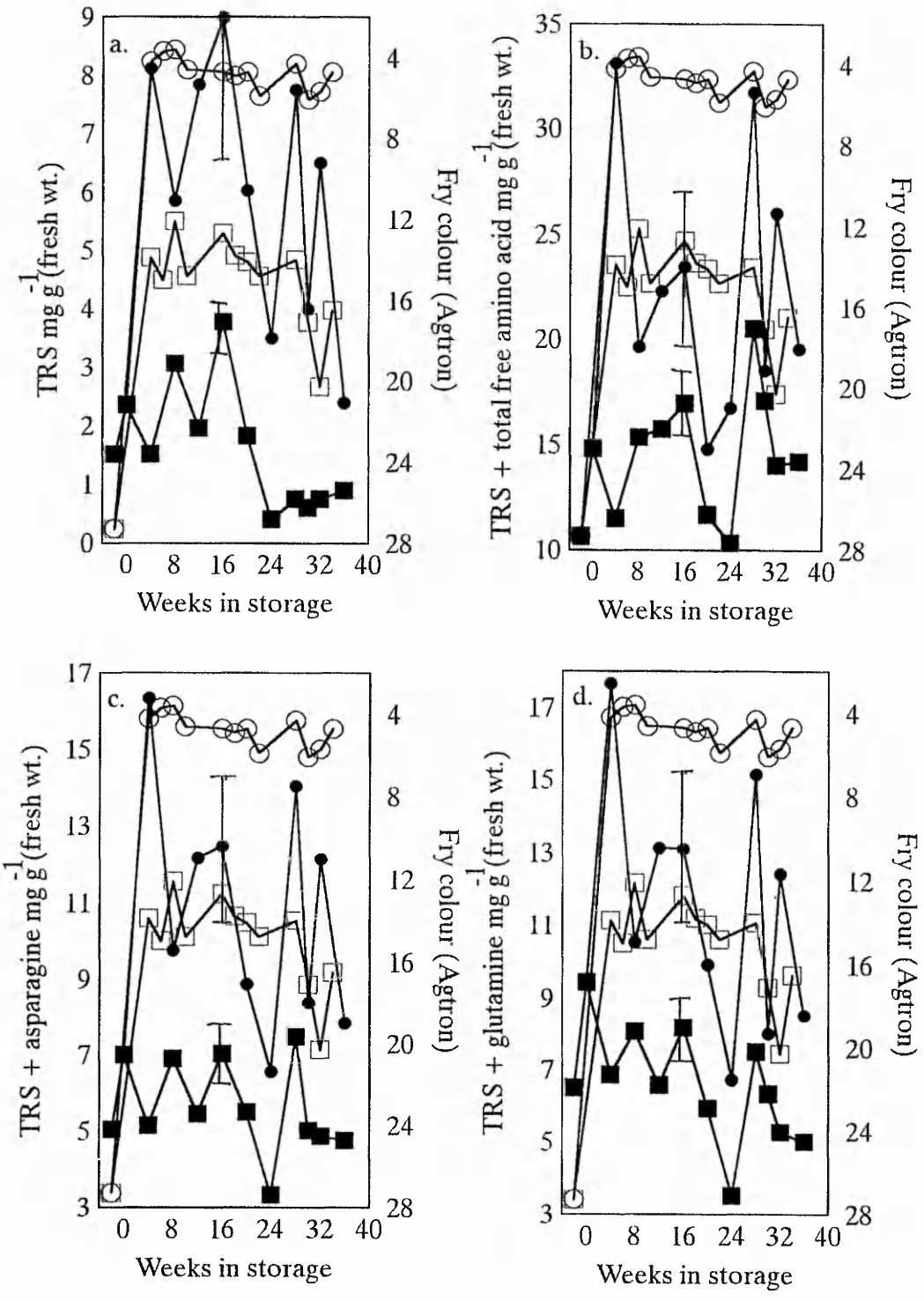
Figures 2.47.a, b, c and d. Fry colour by Agtron 5° (○) and 10°C (□), [a] compared to reducing sugar content, [b] the sum of reducing sugar and amino acid content, [c] the sum of reducing sugars and asparagine and [d] the sum of reducing sugars and glutamine in 5° (●) and 10°C (■) stored Pentland Dell tubers, 1992-93 storage season.



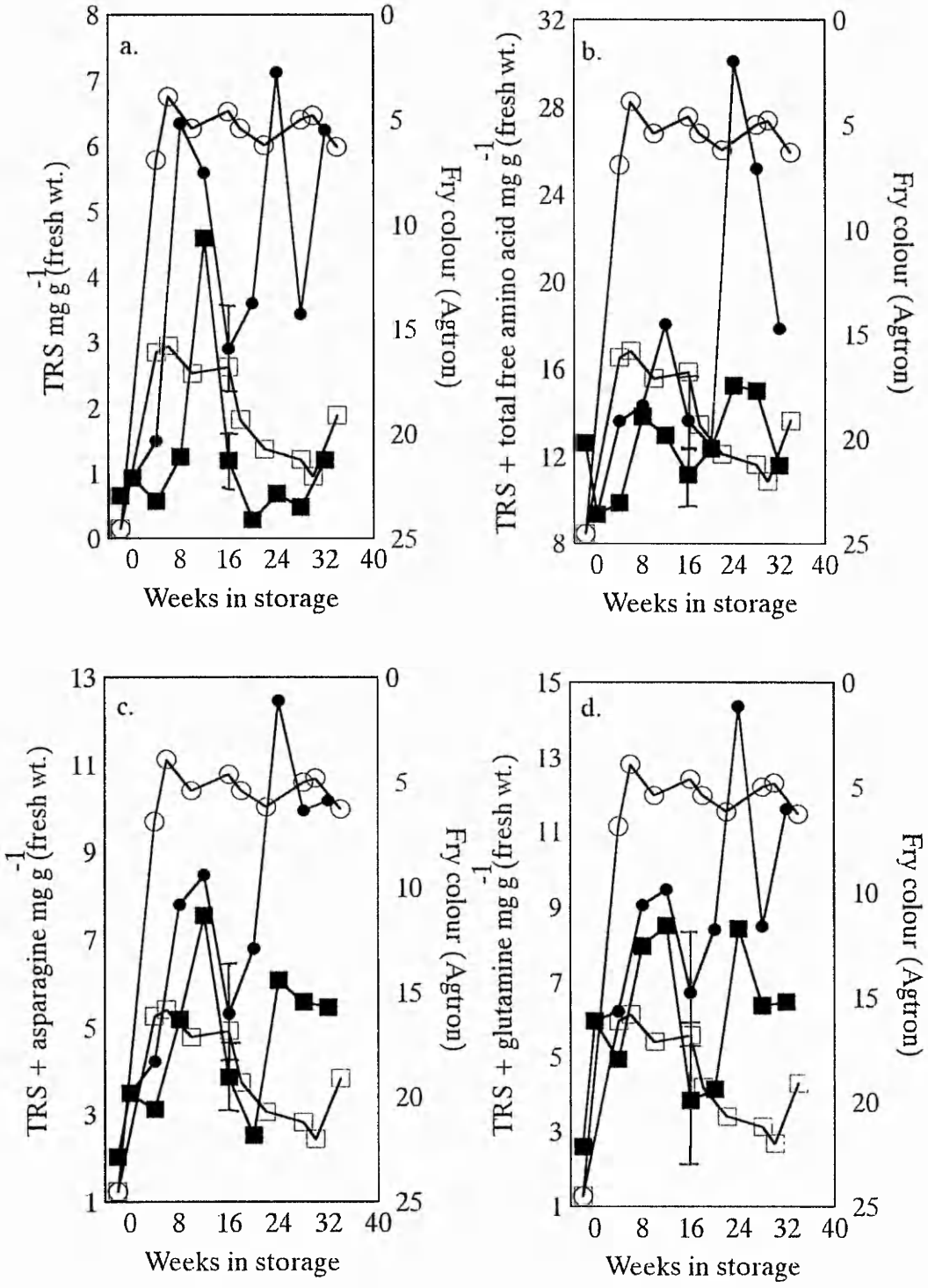
Figures 2.48.a, b, c and d. Fry colour by Agtron 5° (○) and 10°C (□), [a] compared to reducing sugar content, [b] the sum of reducing sugar and amino acid content, [c] the sum of reducing sugars and asparagine and [d] the sum of reducing sugars and glutamine in 5° (●) and 10°C (■) stored Record tubers, 1989-90 storage season.



Figures 2.49.a, b, c and d. Fry colour by Agtron 5° (○) and 10°C (□), [a] compared to reducing sugar content, [b] the sum of reducing sugar and amino acid content, [c] the sum of reducing sugars and asparagine and [d] the sum of reducing sugars and glutamine in 5° (●) and 10°C (■) stored Record tubers, 1990-91 storage season.



Figures 2.50.a, b, c and d. Fry colour by Agtron 5° (○) and 10°C (□), [a] compared to reducing sugar content, [b] the sum of reducing sugar and amino acid content, [c] the sum of reducing sugars and asparagine and [d] the sum of reducing sugars and glutamine in 5° (●) and 10°C (■) stored Record tubers, 1991-92 storage season.



Figures 2.51.a, b, c and d. Fry colour by Agtron 5° (○) and 10°C (□), [a] compared to reducing sugar content, [b] the sum of reducing sugar and amino acid content, [c] the sum of reducing sugars and asparagine and [d] the sum of reducing sugars and glutamine in 5° (●) and 10°C (■) stored Record tubers, 1992-93 storage season.

free amino acids and amides at 10°C did not correspond to a deterioration in fry colour (Figs. 2.48., 2.49., 2.50. and 2.51.). As a result, in the majority of cases reducing sugars alone provided the closest measure of potential fry colour in Record (Table 2.8.).

Table 2.8. Correlation of reducing sugar and amino acid contents with fry colour intensity.

An r value of -1 indicates a perfect fit to fry colour (Agtron units), whilst 0 or a positive number indicates no relationship.

1990-91 season Pentland Dell 5°C	r value	1990-91 season Record 5°C	r value
Colour vs TRS	0.72	Colour vs TRS	0.26
Colour vs TRS+AA	0.49	Colour vs TRS+AA	-0.19
Colour vs TRS+Asn	0.66	Colour vs TRS+Asn	0.27
Colour vs TRS+Gln	0.58	Colour vs TRS+Gln	0.34
Pentland Dell 10°C		Record 10°C	
Colour vs TRS	-0.14	Colour vs TRS	-0.41
Colour vs TRS+AA	-0.47	Colour vs TRS+AA	0.03
Colour vs TRS+Asn	-0.40	Colour vs TRS+Asn	-0.19
Colour vs TRS+Gln	-0.51	Colour vs TRS+Gln	-0.25
1991-92 season Pentland Dell 5°C		1991-92 season Record 5°C	
Colour vs TRS	-0.66	Colour vs TRS	-0.79
Colour vs TRS+AA	-0.65	Colour vs TRS+AA	-0.61
Colour vs TRS+Asn	-0.64	Colour vs TRS+Asn	-0.68
Colour vs TRS+Gln	-0.39	Colour vs TRS+Gln	-0.60
Pentland Dell 10°C		Record 10°C	
Colour vs TRS	-0.29	Colour vs TRS	-0.43
Colour vs TRS+AA	-0.74	Colour vs TRS+AA	-0.45
Colour vs TRS+Asn	-0.41	Colour vs TRS+Asn	-0.61
Colour vs TRS+Gln	-0.40	Colour vs TRS+Gln	-0.54
1992-93 season Pentland Dell 5°C		1992-93 season Record 5°C	
Colour vs TRS	-0.53	Colour vs TRS	-0.82
Colour vs TRS+AA	-0.71	Colour vs TRS+AA	-0.44
Colour vs TRS+Asn	-0.65	Colour vs TRS+Asn	-0.71
Colour vs TRS+Gln	-0.72	Colour vs TRS+Gln	-0.94
Pentland Dell 10°C		Record 10°C	
Colour vs TRS	0.84	Colour vs TRS	-0.40
Colour vs TRS+AA	-0.86	Colour vs TRS+AA	0.68
Colour vs TRS+Asn	-0.99	Colour vs TRS+Asn	-0.20
Colour vs TRS+Gln	-0.75	Colour vs TRS+Gln	-0.32

Due to the absence of any distinct reconditioning effect on either the total free amino acid or individual amide contents of both Pentland Dell and Record tubers , it is likely any fry colour improvements brought about by these treatments are solely due to a decline in reducing sugar content.

2.4. DISCUSSION

The variation in storage trends of amino acid content as demonstrated by both this study and the literature, implies that metabolism of amino acids varies considerably with cultivar and between storage seasons (Fitzpatrick and Porter, 1966; Talley and Porter, 1970; Talley *et al*, 1984; Hart *et al*, 1986; Hart and Cobb, 1988). Fitzpatrick and Porter (1966) demonstrated that a rise of amino acids in Kennebec was not related to a change in total nitrogen content, this implicated protein degradation as the nitrogen source for this increase. It is likely that proteolysis is responsible for free amino acid accumulation in tubers of all cultivars during storage.

In all seasons, the increases of free amino acid pool size exhibited by Pentland Dell were generally greater at 10°C (Figs. 2.06., 2.07., 2.08. and 2.09.), this possibly being explained by an increased rate of metabolism at this temperature relative to tubers stored at 5°C. The lack of any consistent temperature effect on Record amino acid content implied that both protein degradation and synthesis were equally temperature dependent in these tubers. The balance of nitrogen flux rather than the rate of metabolism *per se* is thus likely to be the key factor determining amino acid content. It is probable that reconditioning tubers of both cultivars at 20°C for up to six weeks did not alter the equilibrium of nitrogen flux between proteins and amino acids relative to that in tubers maintained at the standard storage temperature. As a consequence no effect of reconditioning was observed (Figs. 2.06. to 2.08. and 2.10. to 2.12.). In contrast, Fitzpatrick and Porter (1966) observed an escalation of amino acid content as a result of reconditioning Kennebec at room temperature. As sprouting occurred at the end of the 4 week reconditioning period it is likely this amino acid increase was due to proteolysis upon the initiation of sprouting. The balance of nitrogen flow may alter on the breaking of tuber dormancy to favour liberation of free amino acids. The resultant switch of nitrogen flux from equilibrium towards a net degradation of protein

would allow the influence of temperature on metabolism to effect the free amino acid content. This may explain the temperature dependence of the mid to late season upturn of free amino acids in Pentland Dell in 1990-91, 1991-92 and 1992-93 (Figs. 2.07., 2.08. and 2.09.).

The amides asparagine and glutamine generally accounted for between 50% and 90% of the free amino acid content in both Pentland Dell and Record with aspartate and glutamate lesser contributors (Figs. 2.19. to 2.22. and 2.27. to 2.30.), this being consistent with the literature (Talley *et al*, 1964; Fitzpatrick and Porter, 1966; Talley and Porter, 1970; Synge,1977; Davies, 1977; Hart and Cobb, 1988; Cobb *et al*, 1990).

Asparagine and glutamine contents varied with season, cultivar and storage duration (Figs. 2.15. to 2.18. and 2.23. to 2.26.). However, the general pattern of amino acid composition showed considerable similarity between cultivars, these amides invariably forming the largest two fractions of the free amino acid pool (Talley *et al*, 1964; Fitzpatrick and Porter, 1966; Talley and Porter, 1970; Synge,1977; Davies, 1977; Hart and Cobb, 1988; Cobb *et al*, 1990).

The upturn of amide content noted over the latter half of each season in both Pentland Dell and Record was most striking in 1991-92 and 1992-93. This trend was comparable with that observed during prolonged storage of Katahdin by Talley *et al* (1964) and Talley and Porter (1970), hence indicating the importance of the amides as a source of free amino nitrogen to support sprouting in all cultivars. The influence of storage temperature on this amide rise in Pentland Dell was matched by an equal effect on total free amino acid content, consequently temperature did not influence the proportion of the free amino acid pool consisting of the amides (Figs. 2.19. to 2.22.). The study of Pentland Dell by Hart and Cobb (1988) revealed a similar temperature effect throughout storage, the proportion of the free amino acid pool comprised of the

amides remaining at a constant 75% at both 5°C and 10°C. Although generally independent of storage temperature, the degree to which the free amino acid pool was comprised of the amides varied with storage duration (Figs. 2.19. to 2.22. and 2.27. to 2.30.). The mid to late season rise of amide content that occurred for both Pentland Dell and Record in all seasons, generally resulted in an increase of the fraction of the free amino acid pool made up by asparagine and glutamine. This trend implied that the upswing of free amino acids was primarily brought about by an increase of free amides. Consequently, this indicated the importance of asparagine and glutamine as a source of free amino nitrogen to supply sprout growth.

One possible source of this amide increase would be conversion of other amino acids via glutamine synthetase and subsequently asparagine synthetase (Cobb *et al*, 1990). Such a pathway of amide synthesis and conversion would result in formation of glutamine and subsequently asparagine at the expense of other free amino acids unless synchronous protein degradation occurred, this providing a flux of nitrogen into the free amino acid pool. Dilworth and Dure (1978) noted such a pathway of proteolysis followed by flow of nitrogen into glutamine and asparagine during the germination of cotton seeds (*Gossiypium hirsutum)*. In this study, no clear conversion of asparagine to glutamine was observed in any of the seasons for either Pentland Dell or Record (Figs.2.31. to 2.38.), the relative proportions of these amides pointing to tight control of their metabolism. The ratio of asparagine to glutamine was independent of storage temperature and was also unaffected by short term reconditioning treatments, indicating that a possible conversion of glutamine to asparagine by asparagine synthetase would have to be matched by an equal activity of glutamine synthetase regardless of storage temperature.

In addition to the amides, aspartate and glutamate also comprised a large proportion of the free amino acid pool (Table 2.6. and Fig. 2.14), glutamate generally forming the

larger fraction in all seasons for both cultivars. This trend was compatible with the literature so indicating further similarities of amino acid pool composition between cultivars (Talley *et al*,1964; Talley and Porter, 1970; Synge, 1977; Davies, 1977; Talley *et al*, 1984). As with the total free amino acid and amide contents, aspartate and glutamate increased with storage duration (Talley *et al*, 1964; Talley and Porter, 1970), this rise taking place predominantly over the latter half of storage for both Pentland Dell and Record (Figs. 2.39. and 2.41.). The temperature dependence of this upturn in Pentland Dell was possibly an outcome of a greater degree of metabolic activity at 10°C, these amino acids either being produced directly from the degradation of storage protein or by the conversion of other amino acids. As the total free amino acid, glutamine, asparagine, aspartate and glutamate pools of both cultivars all exhibited this mid to late season rise, it is likely the net balance of nitrogen flow altered at this stage in storage to favour protein breakdown. This was probably a consequence of tuber emergence from dormancy. A downward trend of glutamate pool size was noted for both Pentland Dell and Record in the 1990-91 season (Fig. 2.40.). When related to the concomitant increase of amide content, this suggested the possible conversion of glutamate to glutamine by glutamine synthetase.

It is therefore likely that the free amino acid pool size of stored tubers at any given point in storage was controlled by the both the degree of proteolysis and protein synthesis. The composition of the pool, possibly being dictated by the amino acid content of the storage proteins and the degree of amino acid conversion subsequent to proteolysis. The net rate of amidation of free amino acids was probably being the most important aspect of this conversion as it regulates the extent to which amides comprise the free amino acid pool.

Although tuber reducing sugar sugar content has long been assumed by processors to be the sole factor controlling processing potential, studies by Hope *et al* (1960),

Wünsch and Schaller (1972), Hart and Cobb (1988), Cobb *et al* (1990) and Roe *et al* (1990) have suggested that free amino acids also play an important role. In this study, as in that of Hart and Cobb (1988), the total free amino acid content was in excess with respect to the total reducing sugar pool (Table 2.7.). Due to the excessive concentration of free amino acids in tuber tissue, it would be expected that reducing sugars limit the degree of colour production upon processing of both 5° and 10°C stored Pentland Dell and Record.

Studies of the effect of nitrogen fertiliser application on tuber processing potential, have shown that tubers grown under high nitrogen regimes produce darker fry colours upon processing than those from low nitrogen regimes (Hope *et al*, 1960; Hughes and Fuller, 1984; Roe *et al*, 1990). Due to the strong correlation of nitrogen fertiliser application with tuber free amino acid content (Hope *et al*, 1960; Hughes and Fuller, 1984; Millard, 1986; Roe *et al*, 1990) it follows that amino acids may play a synergistic role in colour production (Roe *et al*, 1990). The free amino acid content has been observed to be a factor determining fry colour intensity for Pentland Dell tubers stored at 10°C in all seasons (Figs. 2.43. to 2.47. and Table 2.8.). The accumulation of free amino acids over the latter half of the storage season (1990-91, 1991-92 and 1992-93) in 10°C stored Pentland Dell generally resulted in a greater degree of colour production per unit of reducing sugar (Figs. 2.44., 2.45. and 2.47.), this supporting the possibility of a synergistic effect as advocated by Roe *et al* (1990). Hughes and Fuller (1984) also showed an increased fry colour intensity per unit of reducing sugar in high amino acid content tubers of Record at 10°C, thus implying this effect is not confined to Pentland Dell.

Pentland Dell stored at 5°C and Record at 5°C and 10°C displayed no such influence of amino acid content, reducing sugars alone providing the clearest indicator of potential fry colour (Figs. 2.43., 2.44., 2.45., 2.47., 2.48., 2.49., 2.50., 2.51. and table

2.8.). The poor fry colour exhibited throughout storage by 5°C Pentland Dell was a result of low temperature sweetening (Barker, 1932; Burton, 1982). The lower free amino acid pool size of these tubers over the latter half of the season relative to that of 10°C stored tubers (Figs. 2.07., 2.08. and 2.09.), in conjunction with the high reducing sugar content, lessened the likelihood of an observable effect of amino acids. As no consistent effect of reconditioning has been observed on total free amino acid or amide content for either cultivar, any improvement in fry colour brought about by reconditioning is likely to be due to a decrease in the reducing sugar pool size (Williams and Cobb, 1992).

Record generally displayed a darker fry colour per unit reducing sugar than Pentland Dell. This effect occurred both at harvest and throughout storage, even though Pentland Dell and Record exhibited similar free amino acid pool sizes on entry into storage. Thus, fry colour differences between these cultivars cannot be readily explained by an effect of amino acids (Hughes and Fuller, 1984; Roe *et al*, 1990). The processing method itself may have some bearing on fry colour, in all cases Record being processed as crisps and Pentland Dell as French-fries. The greater surface area to volume ratio of crisped potato would allow more contact of the reactants with high temperature oil during frying, this in conjunction with the higher density of Record tuber tissue possibly resulting in more browning per unit reactant than for Pentland Dell processed as french-fries.

As asparagine and glutamine formed the largest fractions of the free amino acid pool, it would be expected that they influence fry colour production to a similar degree as total free amino acid content (Roe *et al*, 1990). In all seasons studied, the amides were factors involved in the control of fry colour development for 10°C stored Pentland Dell (Figs. 2.43., 2.44., 2.45. and 2.47.). In 1990-91 and 1992-93, both amides exerted a comparable degree of influence on fry colour intensity at 10°C to

that of free amino acid content. The extent of influence of the amides was consistent with the observation that they comprised the bulk of the free amino acid pool over the latter half of each storage season in 10°C Pentland Dell (Figs. 2.19. to 2.22.). A lesser effect of the amides was noted in the 1991-92 season. This was brought about by a decline in the relative proportion of amides over the final eight weeks of the 1991-92 season, hence diminishing their influence on fry colour production (Table 2.8.). Several studies have proposed a prime role for either glutamine or asparagine in fry colour production (Hart and Cobb, 1988; Cobb *et al*, 1990; Khanbari and Thompson, 1993). The concentration of the latter amide increasing throughout storage relative to that of glutamine, possibly due to the action of asparagine synthetase (Hart and Cobb, 1988; Cobb *et al*, 1990). In this investigation however, the lack of such an interconversion of amides (Figs. 2.31. to 2.38.) combined with their similar degrees of influence on fry colour (Table 2.8.), inferred that the amides are of equal importance in determining the processing quality of 10°C Pentland Dell.

The results of this chapter have suggested a degree of protein degradation must occur during tuber storage, this providing a source of free amino acids for the increase which occurs over the latter half of each storage season. The following studies were hence performed in order to examine the factors controlling the size and composition of the free amino acid pool during prolonged storage, these are detailed in chapter 3. The soluble protein pool size was hence examined, in order to assess the degree of protein breakdown and the importance of protein content changes in controlling the free amino acid pool size during prolonged storage. The composition of the tuber soluble protein pool was also examined, the relative importance of the glycoprotein patatin as a source of free amino acids being investigated over the 1991-93 and 1992-93 storage seasons. The degree of tuber proteolytic enzyme activity was determined over the 1992-93 season, proteolysis being related to both total free amino acid and amide pool sizes in order to assess the probable role in controlling both total pool size

and amino acid composition. Control of the proportion of the free amino acid pool consisting of the amides was also examined in the 1992-93 season, the activity of the key enzyme involved in amidation of amino acids; glutamine synthetase being followed throughout storage. This activity was related to that of NADH-GOGAT a key enzyme of deamidation, so providing an overall view of net nitrogen flux between the amides and other amino acids, in order to explain changes in amide content relative to the total free amino acid pool.

2.5. CONCLUSIONS

The following conclusions may be drawn from the results detailed in this chapter.

Firstly, tuber free amino acid content has been shown to be influenced by storage duration, an increase occurring in both Pentland Dell and Record tubers after 24 weeks in storage. This increase which demonstrated temperature dependence in Pentland Dell tubers only, was predominantly due to rises of asparagine and glutamine, although aspartate and glutamate were also shown to rise.

Secondly, asparagine and glutamine comprised between 50 and 90% of the free amino acid pool, this proportion varied with storage duration but generally rose over the latter half of each storage season. Aspartate and glutamate also comprised a large fraction of the free amino acid pool though these totalled less than 20% of the pool. The overall pattern of amino acid pool composition varied little with season or cultivar.

Thirdly, short term reconditioning of tubers does not influence free amino acid content or composition, any effect of reconditioning on fry colour is thus due only to the reduction of reducing sugars generally observed.

Finally, although in excess with respect to reducing sugars, free amino acids in conjunction with reducing sugars influenced the degree of fry colour production in Pentland Dell tubers stored at 10°C. Darker fry colours per unit of reducing sugar occurred over the latter half of storage, this being a result of the increased free amino acid content, probably causing a synergistic effect. Due to their relative concentrations, the amides asparagine and glutamine influenced fry colour intensity to a similar degree as total free amino acid pool size in 10°C stored Pentland Dell tubers,

both amides exerted an equal influence. Amino acid content has not been proved to influence fry colour production for Pentland Dell tubers stored at 5°C or Record tubers stored at 5° and 10°C, in these cases tuber reducing sugar content was the sole observed determinant of potential fry colour intensity.

CHAPTER 3. FACTORS CONTROLLING THE FREE AMINO ACID POOL SIZE AND COMPOSITION OF STORED POTATO TUBERS

3.1. INTRODUCTION

Studies by Tagawa and Okazawa (1955), Fitzpatrick and Porter (1966) and Racusen (1983) have suggested the breakdown of proteins during prolonged storage, producing an increase of free amino acids including that of asparagine and glutamine. Protein degradation has been associated with the breaking of tuber dormancy and the mobilisation of nitrogen reserves providing for subsequent sprout formation (Davies and Ross, 1984). The work of Nowak and Skwiercz (1975) and Davies and Ross (1987) indicated that this trend was consistent with proteolytic enzyme activity, a large upturn in activity being associated with the breakage of dormancy (Nowak and Skwiercz, 1975). However, the studies of Levitt (1954), Cotrufo and Levitt (1958), and Nowak (1977) have implied that protein synthesis may also occur during prolonged storage with low storage temperatures reducing the degree of synthesis (Nowak, 1977) whilst enhancing protease activity (Nowak and Skwiercz, 1975).

Studies by Kapoor, Desborough and Li (1975), Van Gelder and Vonk (1980) and Gorenstein (1988) indicated that the soluble protein pool of potato tubers contained a high proportion of aspartate and glutamate residues. This provided a source of these free amino acids upon proteolysis. Subsequent amidation of the free amino acids probably occurred in order to account for the increase of amides observed by Tagawa and Okazawa (1955), Fitzpatrick and Porter (1966), Hart and Cobb (1988) and Cobb *et al* (1990). Such a conversion of aspartate and glutamate to the amides during tuber storage would be via glutamine synthetase and asparagine synthetase, as observed in seed development and germination (Dilworth and Dure, 1978; Oaks and Ross, 1984) and root nitrogen metabolism in a variety of higher plants (Mack and Tischner, 1990; Vézina and Margolis, 1990).

In this Chapter, factors that play a possible role in the control of tuber amino acid content and composition have been investigated for both Pentland Dell and Record tubers stored at 5° and 10°C. The soluble protein pool size has been examined over 4 storage seasons and compared with free amino acid pool size. The aim being to establish the direction of net nitrogen flow between the protein and free amino acid pools, as a function of both storage duration and temperature. The effect of reconditioning treatments on nitrogen flow were also examined, their effectiveness as a means of altering tuber amino acid content being assessed. The composition of the soluble protein pool has also been examined in order to identify the principal protein species present in stored tubers and the content of the glycoprotein patatin was monitored throughout storage. Patatin was of specific interest due to it's high concentration (Racusen, 1983) and amino acid composition, consisting of a large proportion of aspartate and glutamate residues (Racusen and Foote, 1980). Changes during storage of patatin content were compared to those of the free amino acid pool. Consequently, the relative importance of this protein as a source of free amino acids was assessed.

It is likely that the control of protein degradation by the activity of proteolytic enzymes is a key factor in determining tuber free amino acid content. In this study proteolysis has been measured throughout storage (1992-93 season) in order to confirm it's importance and examine any variations between cultivar and storage temperature. In addition, the substrate specificity of observed proteolytic enzyme activity has been determined in order to assess it's importance in the degradation of various tuber protein fractions. The degree of proteolysis and subsequent amidation of free amino acids are likely to play important roles in determining the proportion of the free amino acid pool consisting of the amides. The key enzyme of amidation, glutamine synthetase has hence been examined in order to determine the degree of conversion into the amides during storage, this being compared to activity of NADH-GOGAT, an important deamidation enzyme.

3.2. MATERIALS AND METHODS

3.2.1. Storage and sampling procedure.

Tubers of the cultivars Pentland Dell and Record were harvested and stored as described in Chapter 2. The freeze-dried tuber extracts used for the determination of amino acid and reducing sugar contents during storage were also utilised for the measurement of soluble protein content. In addition to the 5 tubers of each cultivar and temperature regime removed from storage at each sample date for the extraction of amino acids, reducing sugars and soluble proteins, extra tubers were sampled for the extraction of key enzymes during the 1991-92 and 1992-93 storage seasons.

Throughout the 1991-92 storage season, 3 tubers of both cultivars and temperature regimes were removed from storage at each sample date (including reconditioning treatments) for the extraction and assay of NADH-GOGAT activity. Tubers were transported to Nottingham with those used for metabolite extractions in insulated cold boxes and the enzyme was extracted and assayed as soon as possible on each sample day. During the 1992-93 storage season, 3 tubers of both cultivars and temperature regimes were used at each sample date for extraction and assay of NADH-GOGAT, glutamine synthetase and proteolytic enzyme activities. Additional tubers were used to determine the optimum activity and assay conditions of these enzymes before routine assays were undertaken. Tubers were also used in purification studies of proteolytic enzyme activity. Determination of the optimum conditions for NADH-GOGAT activity was carried out at the beginning of the 1991-92 storage season, whilst optimum conditions for all other enzymes were determined at the start of the 1992-93 season. Characterisation and purification studies of proteolytic enzyme activity were carried out during the 1992-93 storage season and the additional tubers removed from storage as required.

3.2.2. Determination of tuber tissue soluble protein content.

The soluble protein content of tuber tissue was measured using a modified version of the Coomassie Blue dye binding assay of Bradford (1976). The binding of the Coomassie Blue dye to the protein in a sample shifts the dye absorbance maximum from 465nm to 595nm, the absorbance at 595nm being related to protein concentration.

Coomassie Blue reagent was made up as follows: 100mg Electran PAGE Blue G90 dye (BDH) was dissolved in 50ml 95% (v/v) aq. ethanol (diluted from absolute ethanol, SpectrosoL BDH) overnight with stirring in a sealed flask. To this was added 100ml 85% (v/v) phosphoric acid (BDH) and made up to 1 litre with distilled water. The reagent was diluted by 50% and filtered prior to use.

To perform the assay 4mg of freeze-dried tuber extract was dissolved in 10ml pure water (Millipore Milli Q reagent grade) and 100µl of this sample added to 5ml of Coomassie Blue reagent. The solution was gently mixed and allowed to stand at room temperature for 15min. The absorbance was then measured at 595nm against a reagent-distilled water blank using a Perkin Elmer model 550S uv-visible spectrophotometer. Absorbance values were compared to a regression coefficient obtained from a standard curve for bovine serum albumin (Fraction V, Sigma). The soluble protein content of tuber tissue was expressed as mg (protein) g^{-1}(tuber tissue fresh weight).

3.2.3. Analysis of the soluble protein pool.

The composition of the tuber tissue soluble protein pool was examined at the start of the 1991-92 storage season using discontinuous SDS-PAGE (sodium dodecylsulphate polyacrylamide gel electrophoresis) (Laemmli, 1970). Freeze-dried tuber extract (1-25mg) was dissolved in 1ml of 62.5mM Tris-SDS pH 6.8 (sample buffer) and boiled for 4min to denature proteins prior to electrophoresis.

Sample buffer was made up as follows: 25% (v/v) 250mM Tris-SDS stock pH 6.8 containing; Tris base (Sigma) 30.3g l^{-1} and SDS (Sigma) 2g l^{-1}, 2% (w/v) SDS (Sigma), 10% (v/v) glycerol (Sigma), 5% (v/v) 2-mercaptoethanol (Sigma) and 0.1% (v/v) bromophenol blue (Sigma).

To prepare the resolving gel the following solutions were mixed together in a Buchner flask: 19.8ml acrylamide stock containing 29.2% (w/v) acrylamide (Sigma) and 0.8% (w/v) N'-N methylene bisacrylamide (Sigma), 30ml 750mM Tris-SDS stock pH 8.8 containing Tris base (Sigma) 90.8g l^{-1}, SDS (Sigma) 2g l^{-1} and 10.2ml distilled water. The mixture was degassed under vacuum for 15min followed by the addition of 1.5ml freshly prepared 1% (w/v) ammonium persulphate (Sigma) and 15µl N,N,N',N'-tetramethylethylenediamine (TEMED) (Sigma). The mixture was then gently mixed avoiding aeration and poured between the gel plates to a height of 12cm. The gel solution was overlayed with 2-butanol (AnalaR, BDH), this excluded air and produced a flat top to the gel whilst polymerisation took place. The top of the resolving gel was rinsed with 50% (v/v) Tris-SDS stock solution pH 8.8 prior to applying the stacking gel and any excess buffer absorbed with filter paper.

The stacking gel was prepared as follows: 2ml acrylamide stock was mixed with 10ml 250mM Tris-SDS stock pH 6.8 containing Tris base (Sigma) 30.3g l^{-1}, SDS (Sigma) 2g l^{-1} and 8ml distilled water. The mixture was degassed under vacuum for 10min followed by the addition of 0.5ml freshly prepared 2% (w/v) ammonium persulphate (Sigma) and 20µl TEMED (Sigma). The solution was further mixed taking care to avoid aeration and applied to the top of the gel mould containing the well forming comb. The stacking gel was allowed to polymerise and the well forming comb removed, the formed sample wells were then washed with electrode buffer.

Electrode buffer was made up as follows: Tris base (Sigma) 15.5g l^{-1}, glycine (Sigma) 72g l^{-1} and SDS (Sigma) 5g l^{-1}. The pH was adjusted to 8.3 and the solution diluted five-fold before use.

Samples and protein standards (Sigma, Dalton Mark VII-L kit) were applied to the wells using a microlitre syringe. The protein standards consisted of bovine serum albumin (66kDa), ovalbumin (45kDa), glyceraldehyde-3-phosphate dehydrogenase (rabbit muscle, 36kDa), carbonic anhydrase (bovine erythrocytes, 29kDa), trypsinogen (bovine pancreas, 24kDa), trypsin inhibitor (soybean, 20.1kDa) and α-lactalbumin (bovine milk, 14.2kDa).

The gel was run at a constant voltage of 125V at 10°C using a Pharmacia model EPS 300/400 power supply until the bromophenol blue tracking dye was 1cm from the base of the resolving gel. The gel was stained for 2hr in the following solution: 500ml methanol (HPLC Solvent, Fisons), 500ml distilled water, 100ml glacial acetic acid (AnalaR, BDH) and 0.1% (w/v) PAGE Blue 100 (BDH). Following staining the gel was rinsed with distilled water and placed into destaining solution to remove stain unbound to protein. This contained 400ml methanol (HPLC Solvent, Fisons), 500ml distilled water and 100ml glacial acetic acid (AnalaR, BDH).

The protein standards were used to produce a calibration curve for monomeric molecular weight by plotting Rf (band run distance/ bromophenol blue dye front run distance) against Log molecular weight of the protein standards (Fig. 3.01.). The molecular weight composition of the tuber soluble protein pool could be determined using the Rf of tuber protein bands.

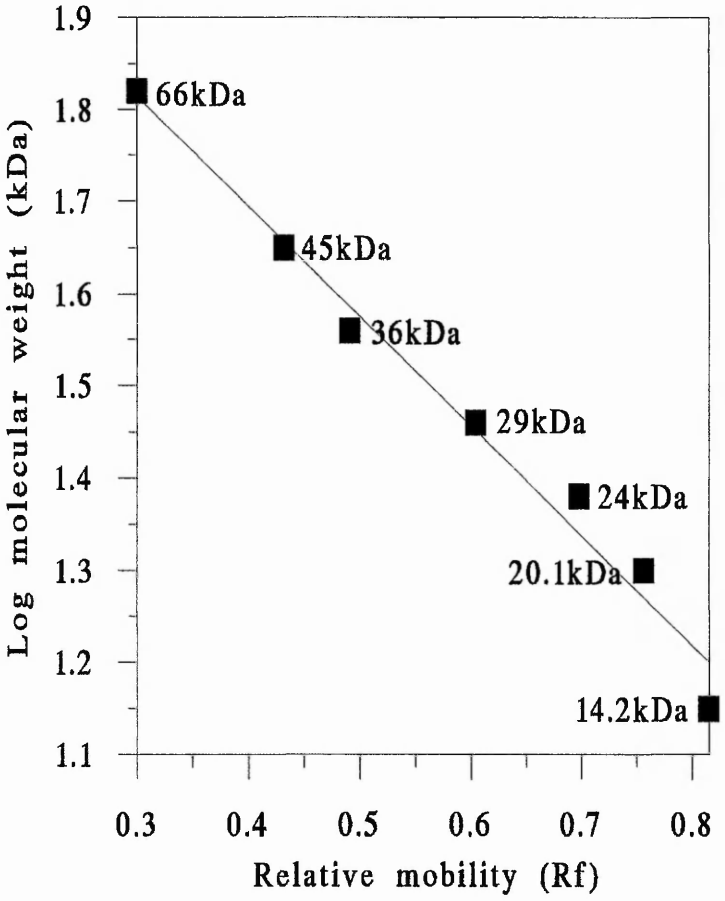


Figure 3.01. A typical calibration curve for monomeric molecular weight determination by SDS-PAGE.

3.2.4. Determination of tuber tissue patatin content.

The patatin content of the tuber tissue soluble protein pool was monitored throughout the 1991-92 and 1992-93 storage seasons using gel filtration liquid chromatography. Freeze-dried potato core tissue (100mg) was suspended in 2ml extraction buffer (Tris base (Sigma) 24.2g l^{-1}, dithiothreitol (Sigma) 15.4mg l^{-1}, the pH was adjusted to pH 7.0 with 1M HCl; AnalaR, BDH). The suspension was filtered through a 0.2µm pore size membrane (Acrodisc) and taken up into a 1ml syringe, taking care to remove all bubbles. The total protein content of the sample was determined by the Coomassie Blue dye binding assay of Bradford (1976).

Proteins were separated using a Beckman System Gold HPLC fitted with a model 116/126 pump and model 106 UV-absorbance detector. The sample (100µl) was injected onto a Phenomenex Biosep SEC S2000 PEEK gel filtration column, and an isocratic buffer system (extraction buffer) used to separate protein fractions at a flow rate of 0.5ml min^{-1}. The absorbance of the eluant was monitored at 280nm and data collected up to an elution time of 35min. The area of the patatin peak was compared to the total peak area so giving a value for patatin as a percentage of total protein. The patatin content was calculated using the total protein content of the sample and results expressed as mg (patatin) g^{-1}(tuber tissue fresh weight).

3.2.5. Measurement of proteolysis in stored potato tubers.

Tuber proteolytic enzyme activity was monitored throughout the 1992-93 storage season using fluorescein isothiocyanate-labelled casein (FITC-casein) as substrate (Twining, 1984). This fluorescent labelled protein was prepared by the following method: 1g of casein (Sigma) was dissolved in 100ml 50mM sodium carbonate buffer pH 9.5 containing 150mM NaCl (Sigma), the pH being readjusted after the casein was dissolved. To this solution was added 40mg FITC (Sigma) and the mixture stirred gently for 1hr at room temperature. The FITC-labelled casein was dialysed twice against 2 litres of distilled water containing 1g l^{-1} activated charcoal (Sigma) at 4°C.

The solution was then dialysed against 2 litres 50mM Tris-HCl pH 8.5 followed by 2 litres 50mM Tris-HCl pH 7.2. The final protein content of the FITC-casein was adjusted to 0.5% (w/v) with 50mM Tris-HCl pH 7.2 and the solution divided into 5ml aliquots. The labelled protein was stored at -20°C until use.

Proteolytic enzyme activity was extracted by a modified version of the papain extraction method of Arnon (1970). Tuber core tissue pooled on an equal weight basis from 3 tubers. The tissue was homogenised in citric acid-disodium hydrogen phosphate buffer pH 6.0 (McIlvaine, 1921 cited by Dawson *et al*, 1969), the buffer containing 100mM citric acid (Sigma) adjusted to pH 6.0 with 200mM disodium hydrogen phosphate (AnalaR, BDH). This extraction buffer also contained 2mM EDTA (dihydrate disodium salt, Sigma), 5mM cysteine (Sigma), 10mM NaCl (Sigma) and 0.1% (v/v) Triton X-100. The homogenate was filtered through two layers of muslin and clarified using an MSE Micro-Centaur centrifuge on high speed setting for 5min to produce a crude enzyme extract. This extract was desalted using a Sephadex G25 gel filtration column (Pharmacia PD10). Crude extract (2.5ml) was applied to the top of the column (equilibrated with extraction buffer) and 1ml fractions collected. Once all the extract had entered the gel bed, further extraction buffer was added so maintaining flow through the column. The protein containing fractions (3,4 and 5) were pooled and used to assay proteolytic enzyme activity.

In order to assay activity, 20µl of enzyme extract was incubated at 30°C with 20µl assay buffer and 20µl FITC-casein. The assay buffer consisted of 100mM citric acid (Sigma) adjusted to pH 6.0 with 200mM disodium hydrogen phosphate (AnalaR, BDH) (McIlvaine, 1921 cited by Dawson *et al*, 1969), 2mM EDTA (dihydrate disodium salt, Sigma), 5mM cysteine (Sigma) and 10mM NaCl (AnalaR, BDH). The assay was performed in the wells of a microtitre plate, the plate being suspended in a water bath at 30°C so that the base of each well was submerged. The reaction was terminated after 2h incubation by the addition of 120µl 5% (w/v) trichloro-acetic acid

(TCA) (AnalaR, BDH) so precipitating the protein in each well . TCA was added to the control (reaction terminated at 0min) wells before the addition of the FITC-casein substrate so no reaction was able to proceed. The reaction mixture was allowed to stand for 1hr after termination, before being withdrawn from each well with a Pasteur pipette and placed in a microfuge tube. The mixture was then spun for 5min in an MSE Micro-Centaur centrifuge in order to sediment the TCA-precipitated protein. Labelled peptide fragments of cleaved FITC-casein were not precipitated by the addition of TCA, their increase could hence be noted over the incubation period so providing a measure of protease activity. A 60µl aliquot of the supernatant was diluted to 3ml with 500mM Tris-HCl pH 8.5 and gently mixed to ensure the entire sample was at the correct pH.

Fluorescence of the sample was determined using a Perkin Elmer fluorescence spectrophotometer with an excitation wavelength of 490nm and emission wavelength of 525nm (slit width 5nm) (Twining, 1984). The change in sample fluorescence over the 2hr incubation period using the control samples as the initial fluorescence was used as the measure of proteolysis, results being expressed as a specific activity ie. relative fluorescence units mg^{-1}(protein) min^{-1}. One relative fluorescence unit being defined as the fluorescence of a 48µM solution of quinine sulphate (Sigma) (Twining, 1984). Protein content of the enzyme extract was measured by the Coomassie Blue dye binding assay (Bradford, 1976). Before this protease assay could be carried out on a routine basis the optimum pH was determined for potato tuber tissue proteolytic enzyme activity.

The effectiveness of this enzyme activity in the breakdown of tuber storage proteins was assessed at the end of the 1992-93 storage season by determining the substrate specificity of the protease activity in 10°C stored Pentland Dell tubers. The activity of a single partially purified extract was measured against several FITC labelled protein substrates: casein (Sigma), bovine serum albumin (Fraction V, Sigma), patatin and a

tuber protein fraction containing several polypeptides between 17 and 25kDa. The tuber proteins were separated by the gel filtration chromatography method used to quantify patatin (described in section 3.2.4.) and collected using a Pharmacia FPLC fraction collector. The 17-25kDa protein fraction was obtained from the peak which eluted subsequent to patatin in this separation procedure. The fractions obtained from several runs were pooled, lyophilised and labelled with FITC by the method of Twinning (1984) as were the commercially obtained non-tuber proteins.

3.2.6. Measurement of glutamine synthetase activity.

Glutamine synthetase activity of potato tuber tissue was determined throughout the 1992-93 storage season using a synthetase assay (Lea, Blackwell, Chen and Hecht, 1990). This assay utilised hydroxylamine as an alternative substrate to ammonia hence producing γ-glutamylhydroxamate. The product could be quantified by reaction with acidified ferric chloride to give a brown colouration that was measured at 540nm.

$$\text{Glu} + NH_2OH + \text{ATP} \xrightarrow{Mg^{2+}} \gamma\text{-glutamylhydroxamate} + \text{ADP} + \text{Pi} + H_2O$$

Longtitudinal cores were removed from 3 tubers and pooled on an equal weight basis. Tissue was then homogenised in extraction buffer (Hirel and Gadal, 1980): 100mM Tris-HCl pH 8.5, 1mM $MgCl_2$ (AnalaR, BDH), 1mM EDTA dihydrate disodium salt (Sigma) and 0.1% (v/v) 2-mercaptoethanol (Sigma). The homogenate was filtered through 2 layers of muslin and then clarified for 5min using an MSE Micro-Centaur centrifuge on high speed setting. This crude extract (2.5ml) was de-salted using a Sephadex G25 gel filtration column (Pharmacia PD10) equilibrated with extraction buffer. Fractions of 1ml were collected and those containing protein (fractions: 3, 4 and 5) were pooled and used to assay glutamine synthetase activity.

The assay was carried out in microfuge tubes incubated at 30°C in a water bath. A 500µl aliquot of enzyme extract was added to 500µl of freshly prepared assay buffer:

100mM Tris-HCl pH 8.5 containing 50mM glutamate sodium salt (Sigma), 5mM hydroxylamine hydrocloride (Sigma), 50mM $MgSO_4$ (AnalaR, BDH) and 20mM ATP (Sigma) from equine muscle. The optimum assay pH and incubation period was determined at the beginning of the 1992-93 storage season, before routine assays were performed. After 1hr the reaction was terminated by the addition of 700µl of the following acidified ferric chloride solution: 0.67M $FeCl_3$ (Sigma), 0.37M HCl (BDH AnalaR) and 20%(w/v) TCA. In control tubes the acidified ferric chloride was added before the enzyme extract so the protein was precipitated immediately with no reaction occurring. Protein was sedimented using an MSE Micro-Centaur centrifuge on high speed setting for 5min. The absorbance of each sample was then measured at 540nm using a Perkin Elmer model 550S UV-visible spectrophotometer. Each sample absorbance was compared with the respective control and the absorbance change related to a calibration curve for γ-glutamylhydroxamate concentration. Glutamine synthetase activities were expressed in the form of specific activity ie. nmoles (γ-glutamylhydroxamate) mg^{-1}(protein) min^{-1}.

3.2.7. Determination of NADH-GOGAT activity.

The enzyme NADH-GOGAT was extracted from tubers throughout the 1991-92 and 1992-93 storage seasons. Activity was determined spectrophotometrically by measuring the oxidation of NADH (nicotinamide adenine dinucleotide, reduced form) to NAD (oxidised form) at 340nm. Fresh tuber basal core tissue was pooled on an equal weight basis from three tubers of both cultivars and storage temperatures at each sampling occasion. The tissue was homogenised in extraction buffer (Hecht, Oelmüller, Schmidt and Mohr, 1988) containing: 100mM KH_2PO_4 (AnalaR, BDH) adjusted to pH 8.5 with 1M KOH (AnalaR, BDH), 0.5mM EDTA dihydrate disodium salt (Sigma), 100mM KCl (AnalaR, BDH), 0.1% (v/v) 2-mercaptoethanol and 0.1% (v/v) Triton X-100 (Sigma). The homogenate was filtered through two layers of muslin and clarified using an MSE Micro-Centaur centrifuge on high speed setting for 5min, so producing a crude extract. During the 1992-93 storage season the crude

extract was further purified by de-salting with Sephadex G25 gel filtration columns (Pharmacia PD10), the protein containing fractions (3,4 and 5) were pooled and used in the assay. The assay was carried out at 30°C in a spectrophotometer cuvette, the absorbance at 340nm being monitored continuously throughout the incubation.

The reaction mixture consisted of 1.4ml 100mM KH_2PO_4 (AnalaR, BDH) adjusted to pH 8.5 with 1M KOH (AnalaR, BDH), 200µl 100mM glutamine (Sigma), 200µl 100mM 2-oxoglutarate (Sigma) and 1mM NADH (Sigma) all made up in the above buffer (Hecht *et al*, 1988). The reaction was started by the addition of 500µl enzyme extract and the absorbance monitored at 340nm using a Perkin Elmer model 550S UV-visible spectrophotometer with the temperature control set at 30°C.

Before this assay could be carried out on a routine basis the optimum conditions were determined for activity from potato tuber tissue. This study performed at the start of the 1991-92 storage season ensured that substrate concentrations were in excess (enzyme-limiting conditions) and the reaction was incubated at the optimum pH. The decrease in absorbance at 340nm was related to the molar extinction coefficient of NADH ($E = 6.22x10^3$ for a 1cm path length), so allowing the rate of NADH oxidation to be calculated. NADH-GOGAT activity was expressed as a specific activity ie. nmoles (NADH) mg^{-1}(protein) min^{-1}.

3.3. RESULTS

3.3.1. The soluble protein content of potato tuber tissue during storage and its relationship to the free amino acid pool.

The soluble protein pool showed differing storage trends over the 4 seasons studied in both Pentland Dell and Record, and when compared to the free amino acid pool varying patterns of nitrogen flux were observed.

Pentland Dell showed an increase of the soluble protein pool in the 1989-90, 1991-92 and 1992-93 storage seasons, this rise being significant at 95% with the exception of 5°C stored tubers in 1991-92 (Figs. 3.02., 3.04. and 3.05.). The majority of the protein accumulation occurred over the first 6 weeks of storage in 1989-90 and 1991-92, this resulting in a large reduction of the free amino acid to soluble protein pool ratio (mg g^{-1} fresh weight basis) (Figs. 3.10. and 3.12.). A slight upturn in protein content also occurred over the latter half of 1991-92, whilst in the 1992-93 season the increase came about between 4 and 33 weeks in storage (Figs. 3.04. and 3.05.). Both of these rises of soluble protein brought about little change in the free amino acid to soluble protein ratio due to accompanying rises of the free amino acid pool (Figs. 2.08. and 2.09.). In contrast to the above upward trends of protein content, a four-fold decline ($p<0.05$) was observed in Pentland Dell in 1990-91 over the first 16 weeks of storage (Fig. 3.03.). This caused a five-fold increase in the free amino acid to soluble protein ratio, so suggesting a flux of nitrogen from soluble proteins to amino acids (Fig. 3.11.).

Record displayed similar trends of soluble protein content to Pentland Dell in all seasons (Figs. 3.06., 3.07., 3.08. and 3.09.). The pool size exhibited a significant ($p<0.05$) rise in 1989-90, 1991-92 and 1992-93 (Figs. 3.06., 3.08. and 3.09.), the early season increase in 1989-90 and 1991-92 resulting in a reduction of the free amino acid to soluble protein ratio (Figs. 3.14. and 3.16.).

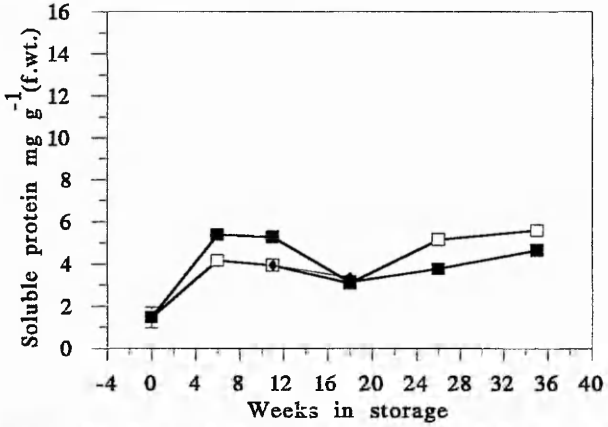


Figure 3.02. The soluble protein content of Pentland Dell tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1989-90 storage season.

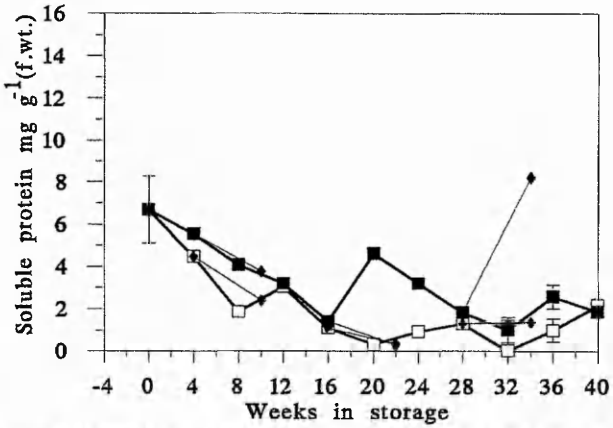


Figure 3.03. The soluble protein content of Pentland Dell tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1990-91 storage season.

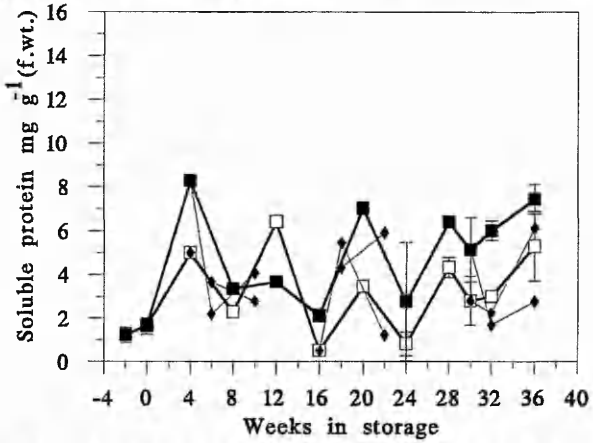


Figure 3.04. The soluble protein content of Pentland Dell tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1991-92 storage season.

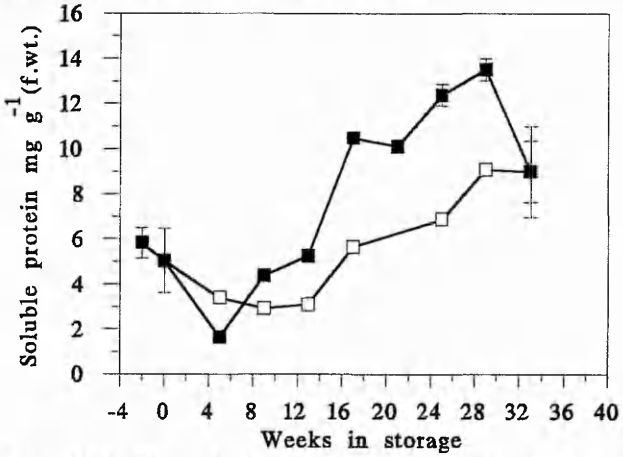


Figure 3.05. The soluble protein content of Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

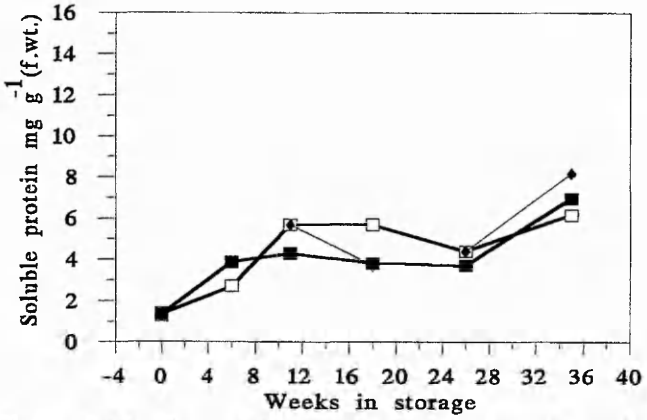


Figure 3.06. The soluble protein content of Record tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1989-90 storage season.

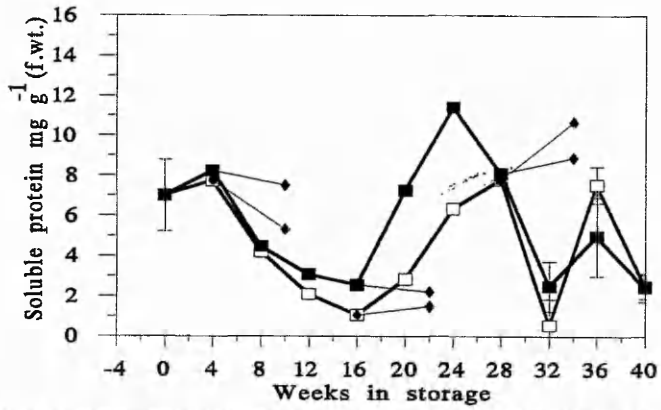


Figure 3.07. The soluble protein content of Record tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1990-91 storage season.

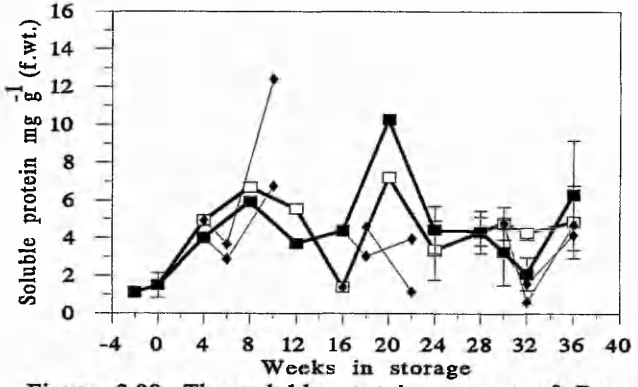


Figure 3.08. The soluble protein content of Record tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1991-92 storage season.

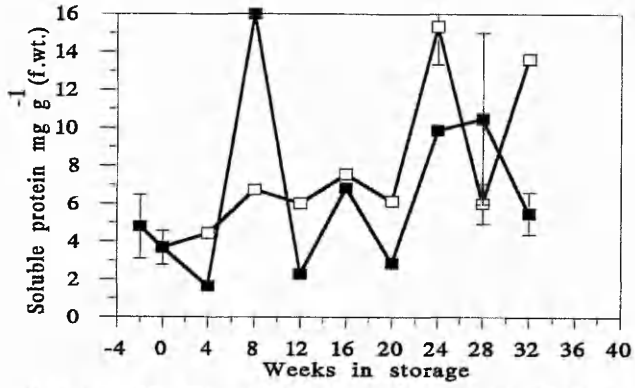


Figure 3.09. The soluble protein content of Record tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

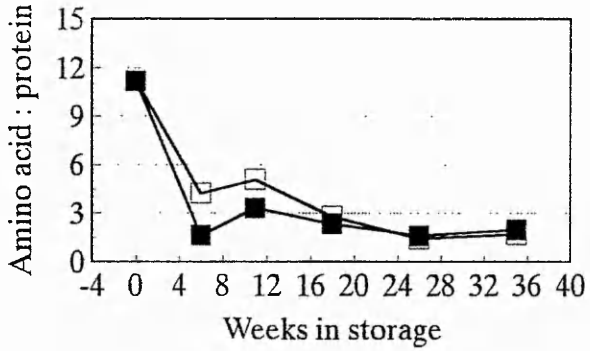


Figure 3.10. The ratio of free amino acid to soluble protein in Pentland Dell tubers (mg g fresh wt. basis) stored at 5°C(□) and 10°C(■),1989-90 storage season.

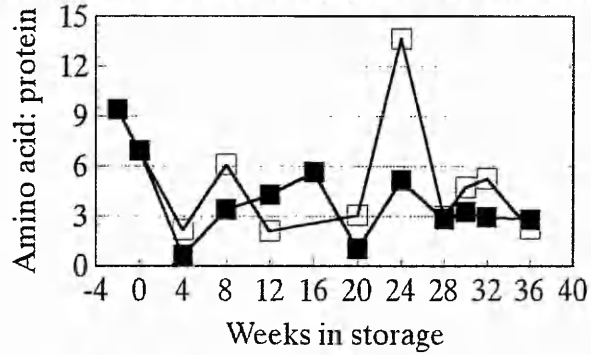


Figure 3.12. The ratio of free amino acid to soluble protein in Pentland Dell tubers (mg g fresh wt. basis) stored at 5°C(□) and 10°C(■),1991-92 storage season.

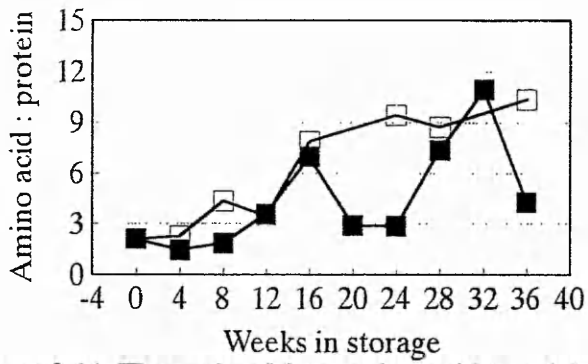


Figure 3.11. The ratio of free amino acid to soluble protein in Pentland Dell tubers (mg g fresh wt. basis) stored at 5°C(□) and 10°C(■),1990-91 storage season.

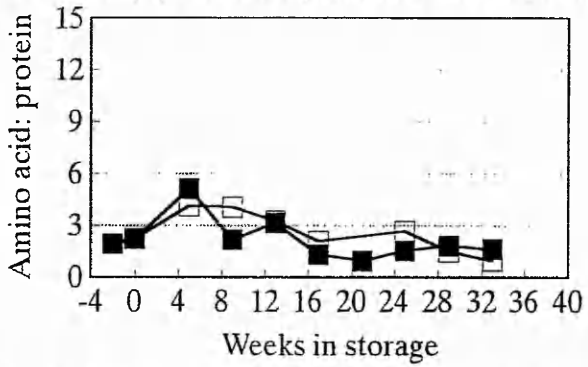


Figure 3.13. The ratio of free amino acid to soluble protein in Pentland Dell tubers (mg g fresh wt. basis) stored at 5°C(□) and 10°C(■), 1992-93 storage season.

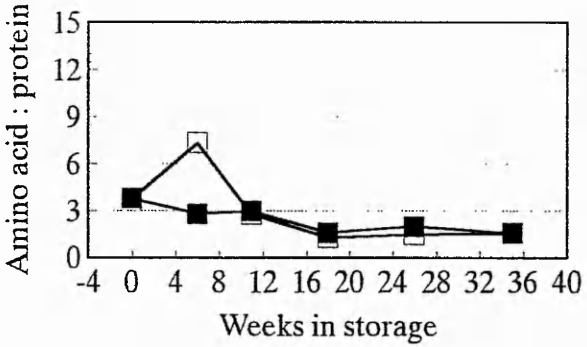


Figure 3.14. The ratio of free amino acid to soluble protein in Record tubers (mg g fresh wt. basis) stored at 5°C(□) and 10°C(■),1989-90 storage season.

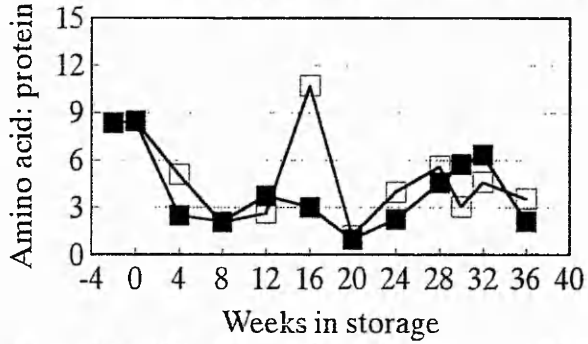


Figure 3.16. The ratio of free amino acid to soluble protein in Record tubers (mg g fresh wt. basis) stored at 5°C(□) and 10°C(■),1991-92 storage season.

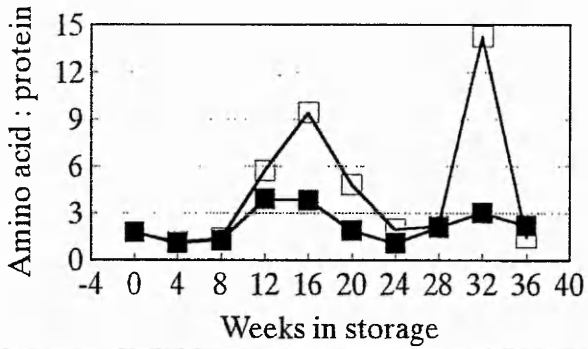


Figure 3.15. The ratio of free amino acid to soluble protein in Record tubers (mg g fresh wt. basis) stored at 5°C(□) and 10°C(■),1990-91 storage season.

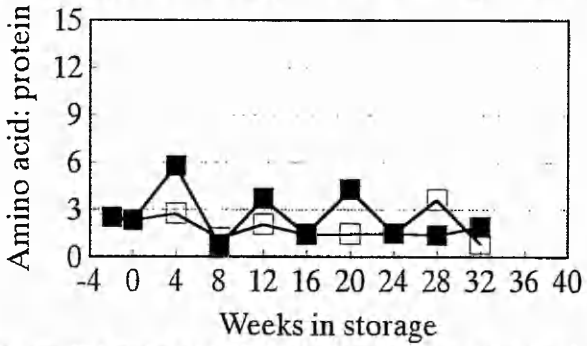


Figure 3.17. The ratio of free amino acid to soluble protein in Record tubers (mg g fresh wt. basis) stored at 5°C(□) and 10°C(■), 1992-93 storage season.

This ratio remained constant throughout 1992-93 as a substantial accumulation of soluble protein was matched by that of the free amino acids (Figs. 2.13., 3.09. and 3.17.). As in Pentland Dell, a four-fold decrease ($p<0.05$) of soluble protein was observed in Record over the first 16 weeks of 1990-91, this causing a rise in the free amino acid to soluble protein ratio (Figs. 3.07. and 3.15.). An upturn and subsequent fluctuation of the soluble protein pool size over the latter half of storage resulted in a fall of the free amino acid to soluble protein ratio from 9:1 to 1:1 at 5°C and 4:1 to 2:1 at 10°C (Figs. 3.07. and 3.15.).

No consistent effect of temperature on soluble protein content was observed for Pentland Dell in all 4 seasons. However, in seasons where soluble protein pool rises occurred such as the 1992-93 season (Fig. 3.05.) and to a lesser extent the latter half of the 1991-92 season (Fig. 3.04.), these increases were approximately 1.5 fold greater at 10°C than at 5°C. Record demonstrated a similar temperature dependence in 1990-91 between 16 and 24 weeks (Fig. 3.07.). No other influence of storage temperature was noted for Record in any of the seasons studied.

Reconditioning treatments did not produce a consistent effect on soluble protein content for either cultivar in any of the 4 seasons studied, in most cases no reconditioning influences being observed. Exceptions to this trend included late-term reconditioning of 10°C stored Pentland Dell in 1990-91, 1991-92 and Record in 1990-91 (Figs. 3.03., 3.04. and 3.07.). In addition, both early and mid-season reconditioning treatments were observed to influence soluble protein pool size for 5° and 10°C stored Record in 1990-91 (Fig. 3.07.).

3.3.2. The soluble protein pool composition of stored potato tubers.

An examination of Pentland Dell and Record extracts by discontinuous SDS-PAGE during the 1991-92 season showed the soluble protein pool to consist of many polypeptides (Fig. 3.18.).

The qualitative composition of the soluble protein pool did not visibly differ between Pentland Dell and Record stored at 5°C and 10°C. The predominant polypeptides (Fig. 3.18.) included a protein with a molecular weight of 44-45kDa, three bands between 20 and 25kDa and a further band at 17-18kDa. Other fainter bands were observed including a band at 87-88kDa which was not observed in all extracts studied.

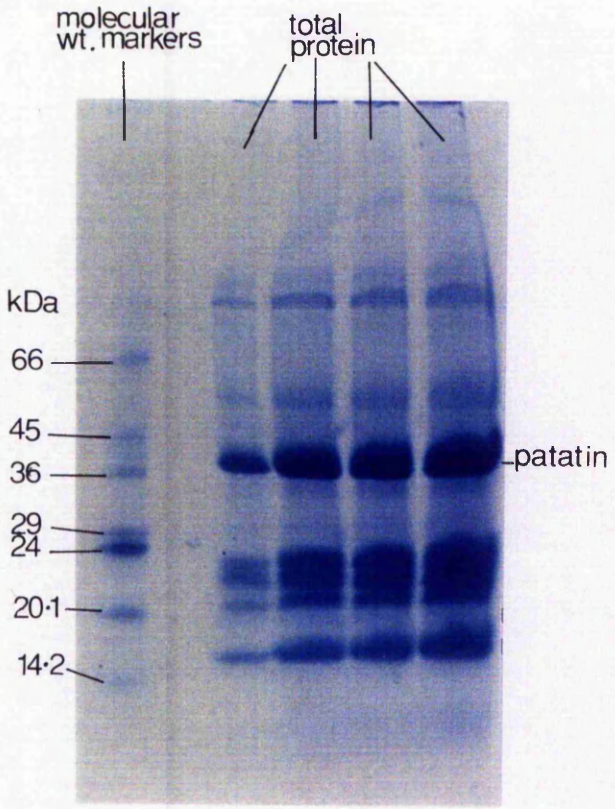


Figure 3.18. Separation of the soluble protein pool of 5°C stored Pentland Dell tubers (1991-92 storage season) by discontinuous SDS-PAGE

Separation of the tuber soluble protein pool by gel-filtration liquid chromatography (using a Phenomenex Biosep SEC S2000 PEEK column) throughout the 1991-92 storage season produced several protein peaks common to each extract, the first fraction to elute being confirmed as the glycoprotein patatin (dimeric molecular

weight 88kDa) due to patatin's lipid acyl hydrolase activity (LAH) and molecular weight (Table 3.1.). When analysed by discontinuous SDS-PAGE (Fig. 3.19.) this fraction was confirmed as the 44-45kDa protein band found in all tuber extracts (Fig. 3.18.). The second fraction eluted from the gel filtration column was shown upon separation by SDS-PAGE to contain the three bands between 20 and 25kDa and the 17-18kDa polypeptide band (Fig. 3.19), as noted in the above SDS-PAGE studies of tuber extracts (Fig.3.18.). This gel filtration fraction was observed to inhibit trypsin activity thus confirming the presence of a proteinase inhibitor (Table 3.1.).

Table 3.1. The lipid acyl hydrolase and trypsin inhibitor activities of tuber protein fractions separated by gel-filtration liquid chromatography.

Protein fraction N^{o} and monomeric range	LAH activity µmoles (p-nitrophenol) mg^{-1}(protein) min^{-1}	Trypsin inhibition %
Fraction 1. 44-45kDa	0.27	4.32
Fraction 2. 17-25kDa	0.00	62.98

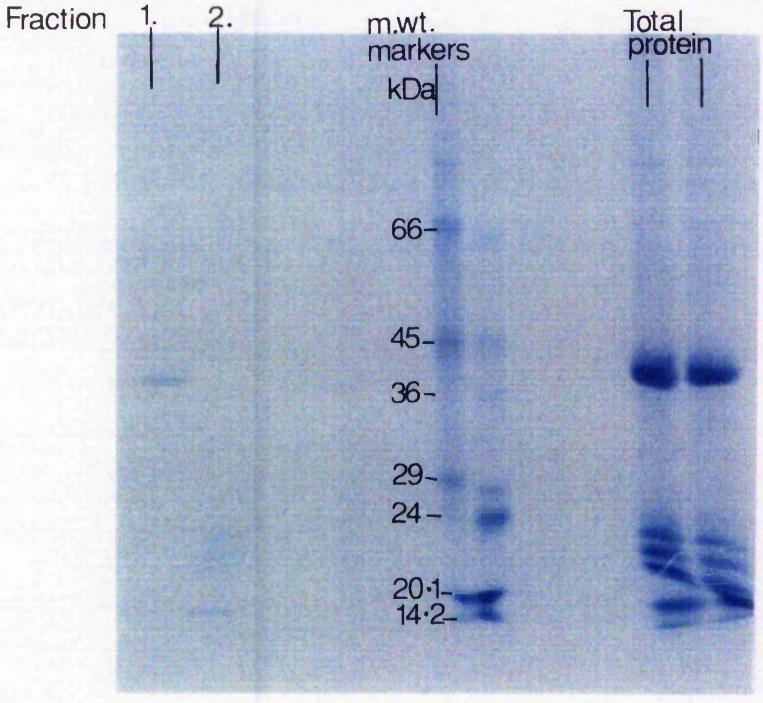


Figure 3.19. SDS-PAGE separation of protein fractions obtained by gel filtration chromatography of Pentland Dell tuber extract (5°C stored, 1991-92 season)

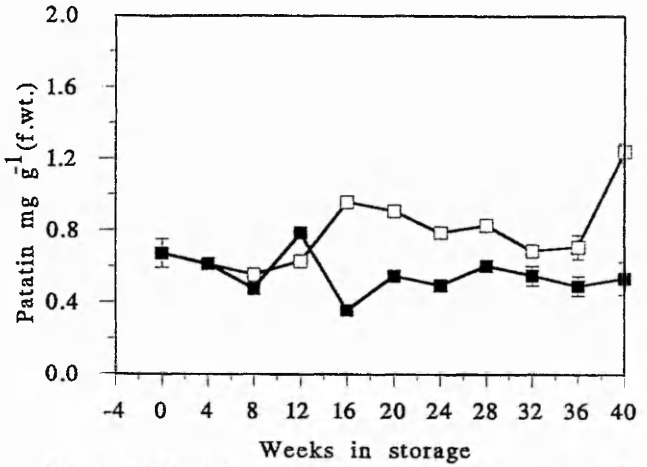


Figure 3.20. The patatin content of Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1991-92 storage season.

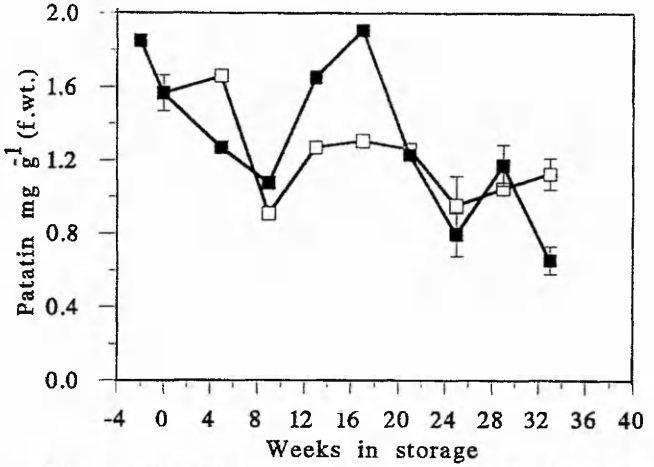


Figure 3.21. The patatin content of Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

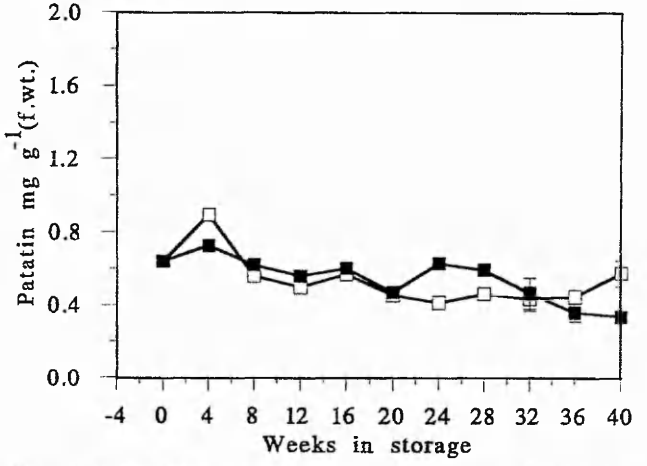


Figure 3.22. The patatin content of Record tubers stored at 5°C(□) and 10°C(■), 1991-92 storage season.

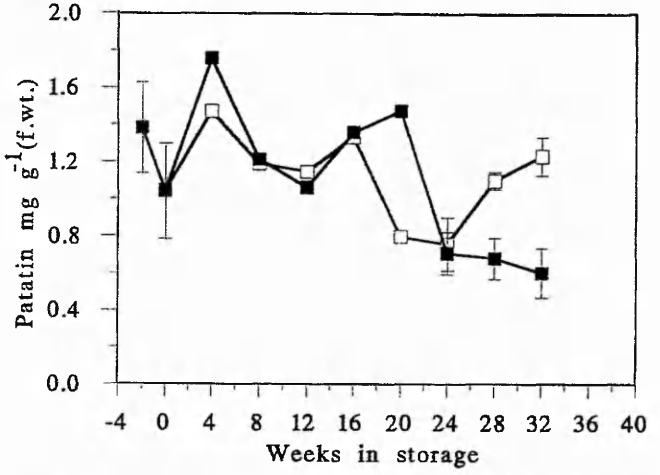


Figure 3.23. The patatin content of Record tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

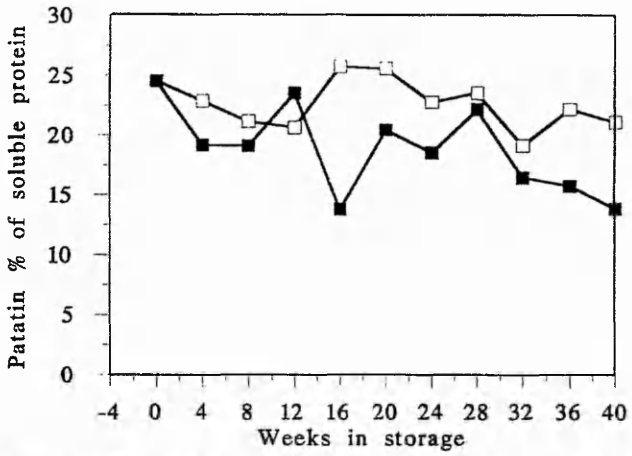


Figure 3.24. Patatin content as a percentage of soluble protein in Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1991-92 storage season.

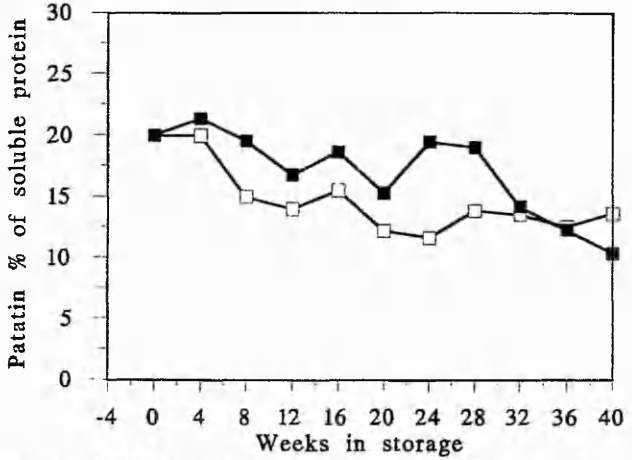


Figure 3.26. Patatin content as a percentage of soluble protein in Record tubers stored at 5°C(□) and 10°C(■), 1991-92 storage season.

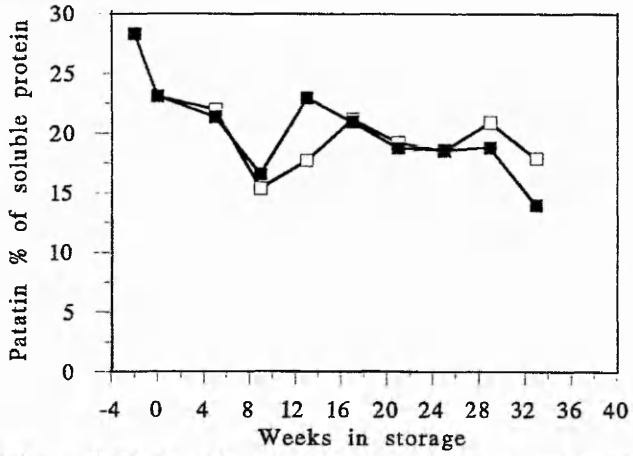


Figure 3.25. Patatin content as a percentage of soluble protein in Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1991-92 storage season.

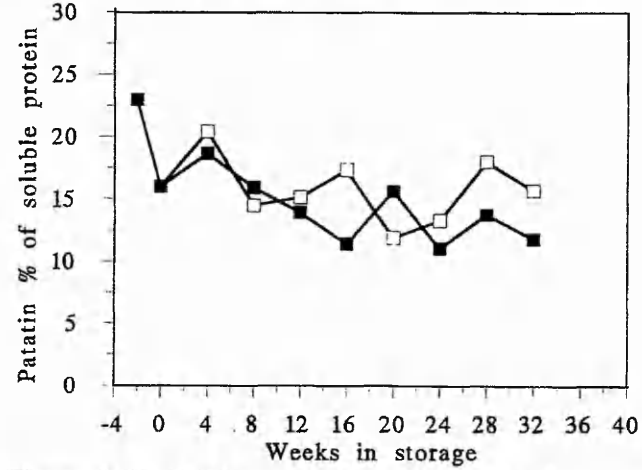


Figure 3.27. Patatin content as a percentage of soluble protein in Record tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

Gel filtration chromatography was used to study changes in the tuber patatin pool during the 1991-92 and 1992-93 storage seasons. In the 1991-92 season, the patatin content of both Pentland Dell and Record stored at 5° and 10°C remained relatively stable at between 0.4 and 0.8 mg (patatin) g^{-1}(fresh weight) throughout most of the season (Figs. 3.20. and 3.22.). Patatin comprised between 25% (w/w) and 15% (w/w) of the total soluble protein pool in Pentland Dell, the proportion remaining stable at 5°C, though falling slightly at 10°C, particularly over the latter half of the season (Fig. 3.24.). In Record, patatin fell as a proportion of the total protein pool from 20% (w/w) to 12% (w/w) due to an increase in soluble protein (Fig.3.26.).

The patatin pool of both cultivars was generally greater in size throughout the 1992-93 storage season than in the previous year. A clear decline in patatin ($p<0.05$) was noted throughout storage in both Pentland Dell and Record at 5° and 10°C (Figs. 3.21. and 3.23.). As a result of this trend and the increase of soluble protein content, patatin fell as a proportion of the soluble protein pool in both Pentland Dell (from 28% to 15% at 10°C and from 28% to 18% at 5°C) and Record (from 23% to 12% at 10°C and from 23% to 16% at 5°C). The decrease of patatin and it's ensuing proportion of the soluble protein pool was slightly greater at 10° than 5°C (Figs. 3.21., 3.23., 3.25. and 3.27.).

3.3.3. Proteolysis in stored potato tubers.

Protease activity in tuber tissue showed a broad pH optimum at around pH 6.0 (Fig. 3.28.), as a result all routine assays throughout the 1992-93 storage season were carried out at this pH.

Proteolytic enzyme activity showed a marked early season decline in both Pentland Dell and Record at 5° and 10°C (Figs. 3.29. and 3.30.). From 8-9 to 20-21 weeks, proteolytic activity remained at a minimal level with no significant ($p>0.05$) temperature difference in either cultivar. Subsequently a rise in activity took place in

both cultivars, the upturn showing temperature dependence ($p<0.05$) in Pentland Dell only (Figs. 3.29. and 3.30.). Following this upswing, activity fluctuated from 25 weeks until the end of storage in Pentland Dell, whereas in Record a decline occurred after 24 weeks (Figs. 3.29. and 3.30.). After 24-25 weeks, proteolytic activity demonstrated temperature dependence in both cultivars.

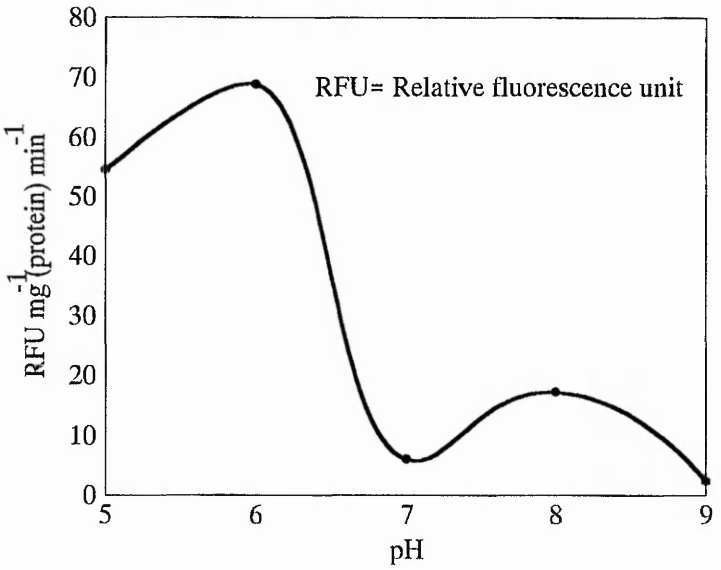


Figure 3.28. The pH optimum of proteolytic enzyme activity from Pentland Dell tuber tissue, 1992-93 storage season.

The seasonal pattern of proteolytic enzyme activity showed a relationship with the total free amino acid pool size in both Pentland Dell and Record stored at 5° and 10°C (1992-93 storage season) (Figs. 3.29., 3.30. and Table 3.2.).

Although the early decline in proteolysis could not be related to the free amino acid pool in either cultivar, the subsequent basal level of activity from 8-9 to 20-21 weeks storage coincided with a low pool size at both 5° and 10°C (Figs. 3.29. and 3.30.). An exception to this trend was the peak of total free amino acid pool size at 13 weeks in 10°C stored Pentland Dell, this not corresponding with the low proteolytic activity. High levels of activity after 20-21 weeks were concurrent with a late season rise of the total free amino acid pool in both Pentland Dell and Record. In Pentland Dell, this

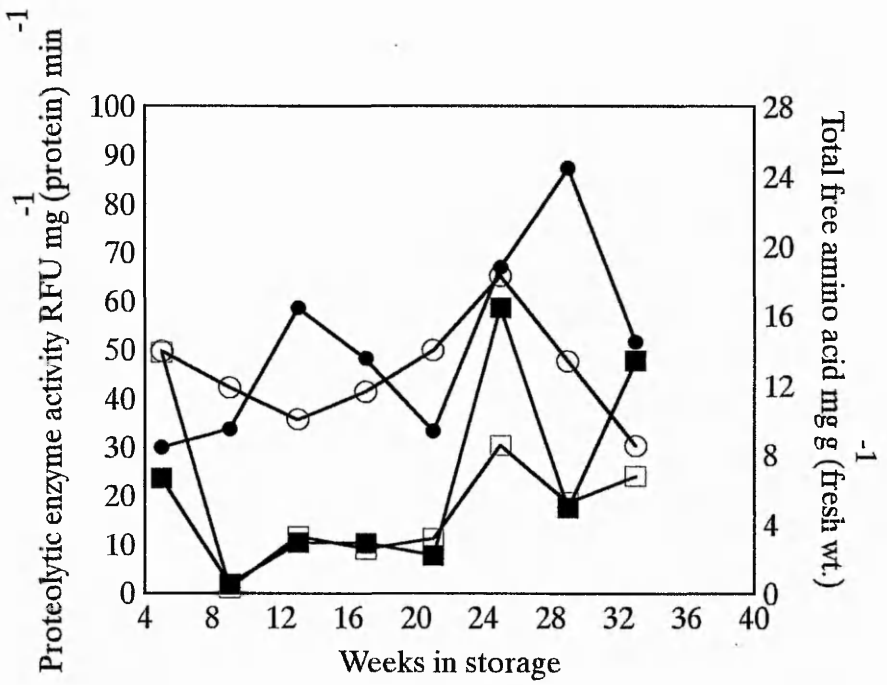


Figure 3.29. The proteolytic enzyme activity at 5°C(□) and 10°C(■), correlated to total free amino acid content of 5°C(○) and 10°C(●) stored Pentland Dell tubers, 1992-93 storage season

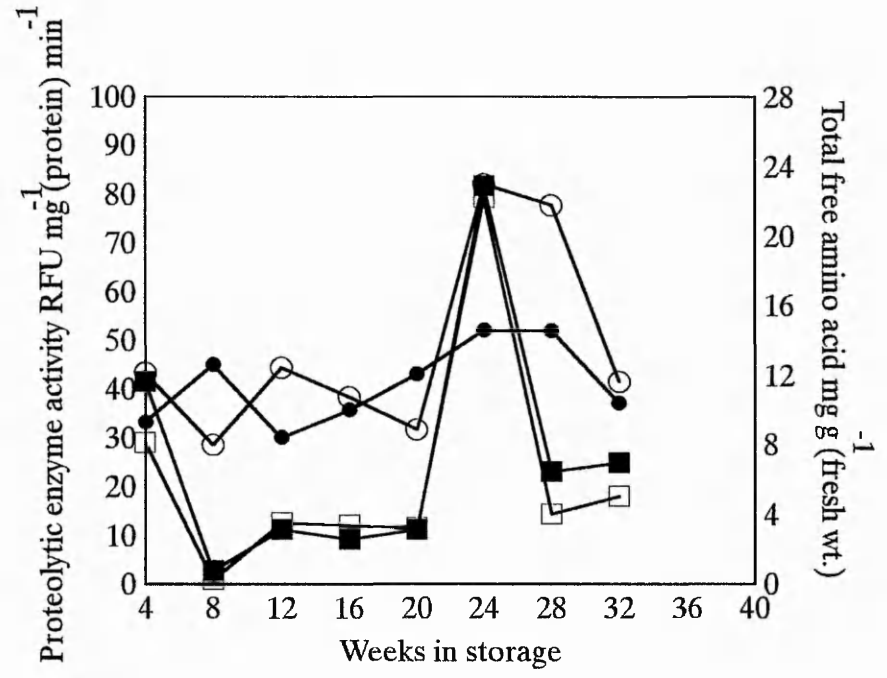


Figure 3.30. The proteolytic enzyme activity at 5°C(□) and 10°C(■), correlated to total free amino acid content of 5°C(○) and 10°C(●) stored Record tubers, 1992-93 storage season

upturn of free amino acids was more pronounced at 10°C than 5°C due to the higher rate of proteolysis (Fig. 3.29.). In Record however, the seven-fold increase of proteolytic activity was not influenced by storage temperature. In contrast, the total free amino acid pool exhibited only a small increase at 10°C compared to that at 5°C (Fig. 3.30.). Regression analysis of the relationship between free amino acid content and proteolytic enzyme activity over the entire storage season, showed a positive correlation between enzyme activity and pool size in both Pentland Dell and Record at 5° and 10°C (Table 3.2.).

Table 3.2. Correlation of proteolytic enzyme activity with total free amino acid and amide contents by regression analysis.

An r value of one indicates a perfect fit whereas zero no relationship, ie. the closer to one the stronger the relationship.

Pentland Dell	5°C	10°C
Total free amino acid	0.37	0.34
Amides (Asn + Gln)	0.58	0.88
Record	5°C	10°C
Total free amino acid	0.70	0.40
Amides (Asn + Gln)	0.71	0.58

Proteolytic enzyme activity also exhibited a strong relationship with the amide fraction of the free amino acid pool (Table 3.2.) for both Pentland Dell and Record during the 1992-93 storage season (Figs. 3.31. and 3.32.). From 8-9 to 20-21 weeks, a minimal rate of proteolysis was reflected by a low concentration of the amides asparagine and glutamine in tuber tissue of both cultivars. The late season upswing of proteolytic activity in both cultivars at 5° and 10°C was matched by a rise in amide content, protein breakdown thus playing a major role in controlling the amide pool as well as the total free amino acid content. This increase in the amide content from 21 weeks in Pentland Dell tubers displayed a marked temperature dependence, tubers stored at

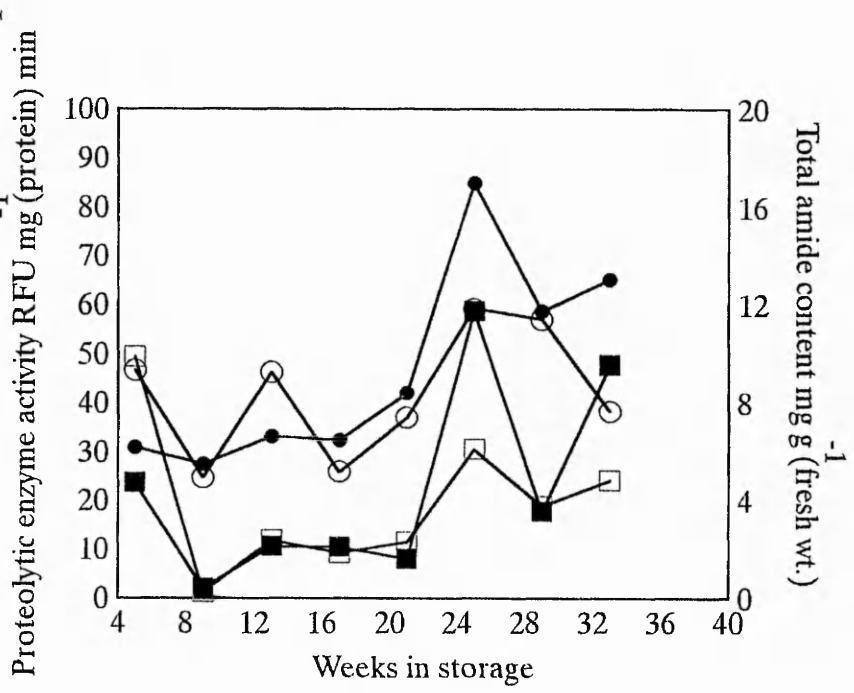


Figure 3.31. The proteolytic enzyme activity at 5°C(□) and 10°C(■), correlated to total amide content of 5°C(○) and 10°C(●) stored Pentland Dell tubers, 1992-93 storage season

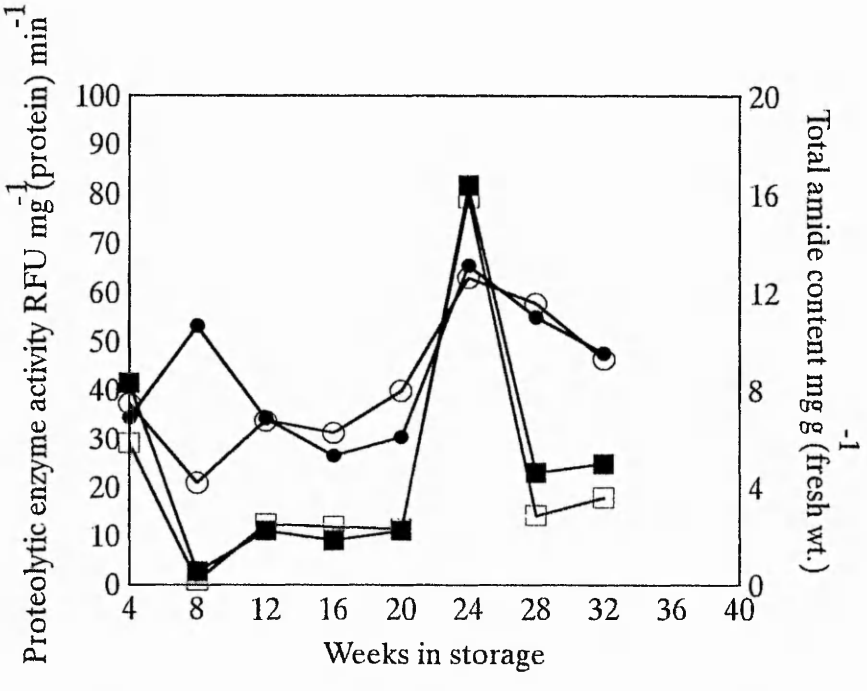


Figure 3.32. The proteolytic enzyme activity at 5°C(□) and 10°C(■), correlated to total amide content of 5°C(○) and 10°C(●) stored Record tubers, 1992-93 storage season

10°C exhibiting a higher rate of proteolysis and hence greater amide pool size than those stored at 5°C (Fig. 3.31.). No temperature influence was noted on the late season upturn of amide content in Record during 1992-93, this trend corresponding with the lack of temperature dependence observed for proteolytic enzyme activity at this stage in storage (Fig. 3.32.). Regression analysis of the relationship between amide content and proteolytic enzyme activity demonstrated a stronger correlation between enzyme activity and pool size than that observed for the total free amino acid pool in both cultivars, an exception being Record tubers stored at 5°C (Table 3.2.).

An examination of the substrate specificity of tuber tissue proteolytic enzyme activity showed that the activity had a pronounced specificity for tuber proteins (FITC labelled) as substrates (Table 3.3.). Of the proteins studied, the enzyme extract showed greatest activity against patatin, this being over three-fold that observed against the 17-25kDa tuber protein fraction. Moderate activity was measured from the same extract against FITC-casein, although this was only approximately one two-hundredth of that observed against patatin. In contrast, no proteolytic enzyme activity was observed against FITC-bovine serum albumin (Table 3.3.).

Table 3.3. The substrate specificity of tuber tissue proteolytic enzyme activity.

1 unit activity = 1 RFU mg^{-1}(protein) min^{-1}

Protein substrate (FITC labelled)	Proteolytic activity units mg^{-1}(protein substrate) $\bar{x}$ (n=8) ±S.D.
patatin	2040.21 ± 38.41
17-25kDa tuber protein fraction	590.87 ± 98.49
casein	1.93 ± 1.64
bovine serum albumin (fraction V)	no activity

3.3.4. Glutamine synthetase and NADH-GOGAT activity of tuber tissue during prolonged storage.

From initial studies carried out at the start of the 1992-93 storage season, the enzyme glutamine synthetase was observed to have a pH optimum between pH 8.0 and 9.0 (Fig. 3.32.), hence all subsequent assays were carried out at pH 8.5.

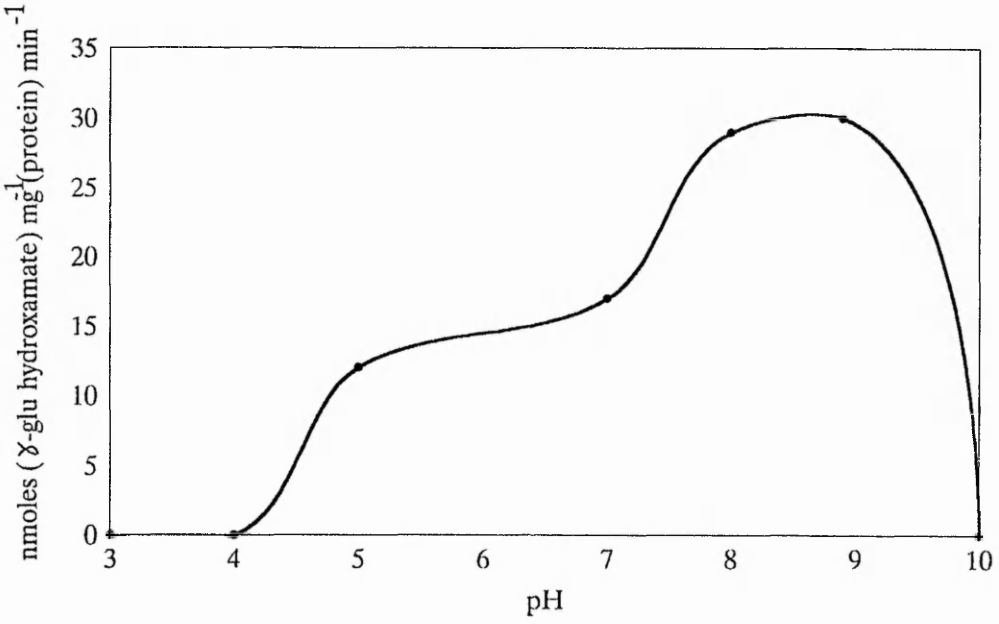


Figure 3.33. The pH optimum of glutamine synthetase activity from Pentland Dell tubers stored at 5°C, 1992-93 storage season.

Glutamine synthetase activity showed marked fluctuation in Pentland Dell throughout 1992-93 although the underlying trend was of a two-fold decline between 9 and 33 weeks in storage (Fig. 3.34.). In Record however, the overall trend of glutamine synthetase activity was relative stability with the exception of a large peak in activity at 16 weeks in storage (Fig. 3.35.). The seasonal pattern of activity was independent of storage temperature in both cultivars, although fluctuation was generally greater in 10°C stored tubers (Figs. 3.34. and 3.35.).

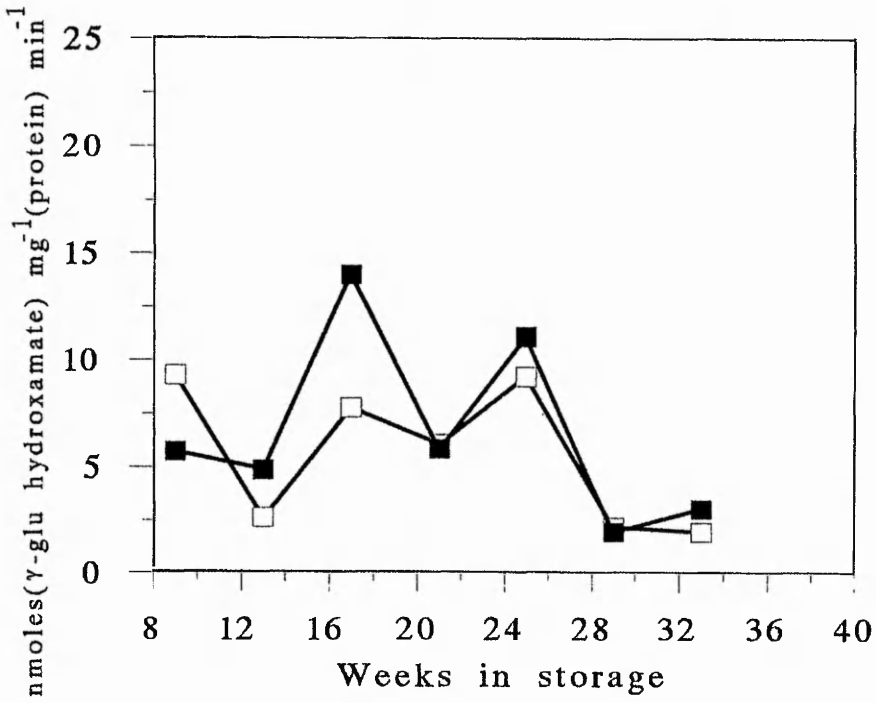


Figure 3.34. The glutamine synthetase activity of tissue from Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

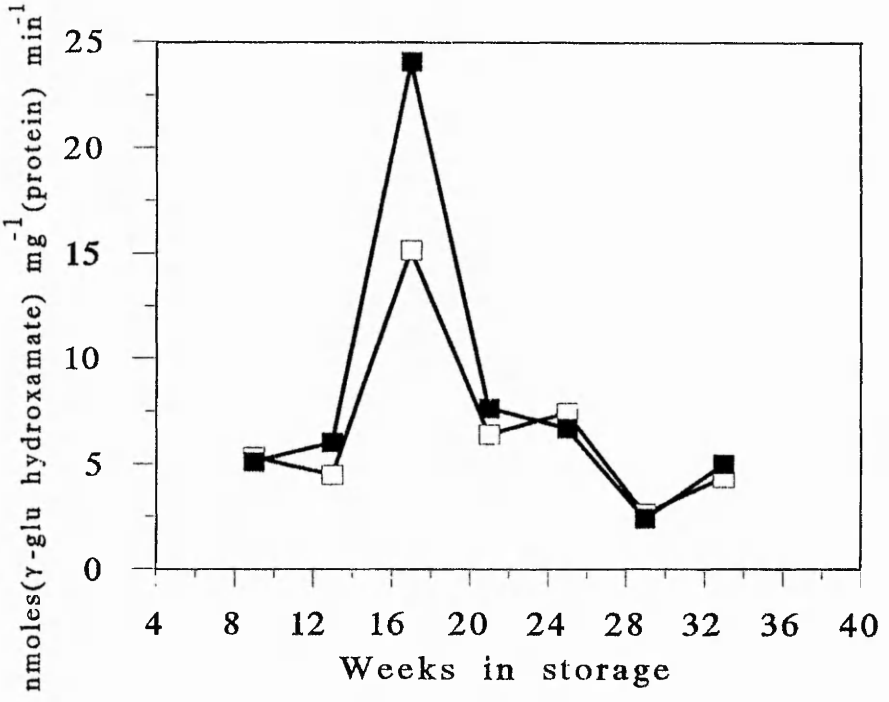


Figure 3.35. The glutamine synthetase activity of tissue from Record tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

Initial studies of NADH-GOGAT activity from 5°C stored Pentland Dell tubers at the start of 1991-92, showed the enzyme to have a broad pH optimum (Fig. 3.36.). All subsequent assays of NADH-GOGAT activity were carried out at pH 8.5.

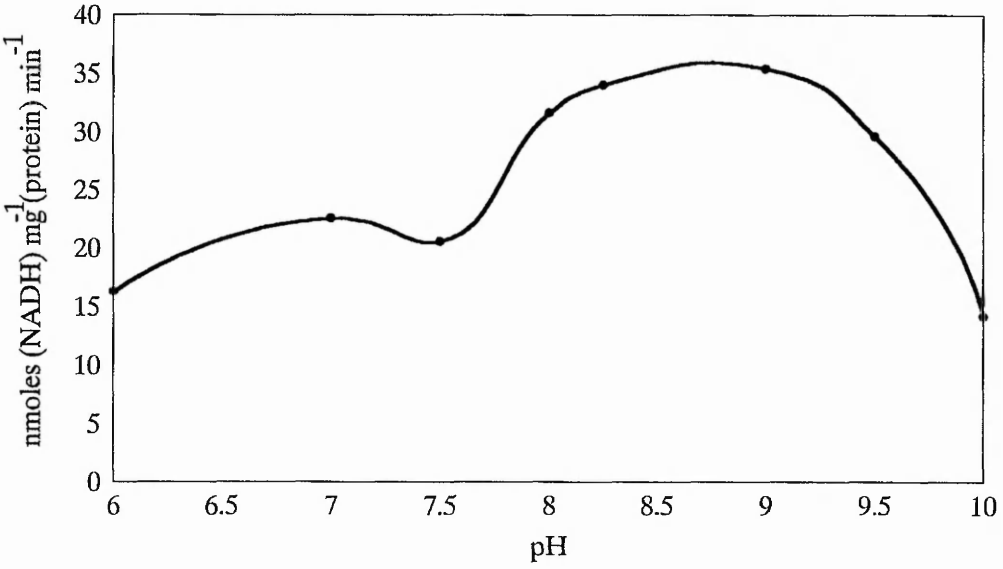


Figure 3.36. The pH optimum of NADH-GOGAT activity from Pentland Dell tubers stored at 5°C, 1991-92 storage season.

The activity of NADH-GOGAT displayed differing trends in the 2 seasons studied (1991-92 and 1992-93). A high rate of NADH-GOGAT activity was exhibited in early storage (4 weeks) by both Pentland Dell and Record in 1991-92, the greatest activity ($p<0.05$) being displayed by tubers stored at 10°C (Figs. 3.37. and 3.38.). Activity subsequently underwent a dramatic drop in both cultivars between 4 and 8 weeks. Following this decline, activity remained at a minimal rate until 16 weeks. Between 16 and 20 weeks NADH-GOGAT activity rose in both cultivars at 5° and 10°C, the rate then fluctuating until the end of storage. Following the initial decline of activity between 4 and 8 weeks, no influence of storage temperature was noted for either cultivar. Early and late season reconditioning treatments also had negligible impact on the rate of NADH-GOGAT activity. In contrast, mid-term reconditioning had an effect on activity by maintaining a minimal level of below 50 nmoles (NADH)

mg^{-1}(protein) min^{-1}after 16 weeks when the rate increased in non-reconditioned tubers (Figs. 3.37. and 3.38).

During 1992-93, NADH-GOGAT activity in both cultivars (partially purified extract) rose between three and four-fold over the first half of the season at 5°C (Figs. 3.39. and 3.40.). Activity increased to a similar degree in tubers stored at 10°C, although a maximum rate was not reached until 28-29 weeks with the exception of a peak in activity in Pentland Dell at 13 weeks (Figs. 3.39. and 3.40.). A subsequent late season downturn in the rate of NADH-GOGAT activity occurred in both Pentland Dell (35% at 5°C and 57% at 10°C) and Record (79% at 5°C and 53% at 10°C) (Fig. 3.40.).

Comparison of the rates of glutamine synthetase and NADH-GOGAT activity throughout the 1992-93 storage season has shown the following trends in enzymic regulation of the glutamine pool size. In both Pentland Dell and Record a decline in the ratio of glutamine synthetase to NADH-GOGAT activity (specific activity basis) was noted until 28-29 weeks at 5° and 10°C (Figs. 3.41. and 3.42). This trend was caused by an overall decrease of glutamine synthetase activity (Figs. 3.34. and 3.35.) coupled to an increase in activity of NADH-GOGAT (Figs. 3.39. and 3.40.). As a result, the balance of glutamine metabolism was in the direction of amino acid amidation, this trend of glutamine synthesis undergoing a temperature independent decline as the season progressed. Net deamidation was observed from 29 weeks until the end of storage in Pentland Dell at 5°C, whereas at 10°C, tubers displayed only a brief period of net deamidation at around 29 weeks (Fig. 3.41.). In Record the ratio underwent a large upturn between 28 and 32 weeks, this suggesting a switch from deamidation to glutamine synthesis (Fig. 3.42.).

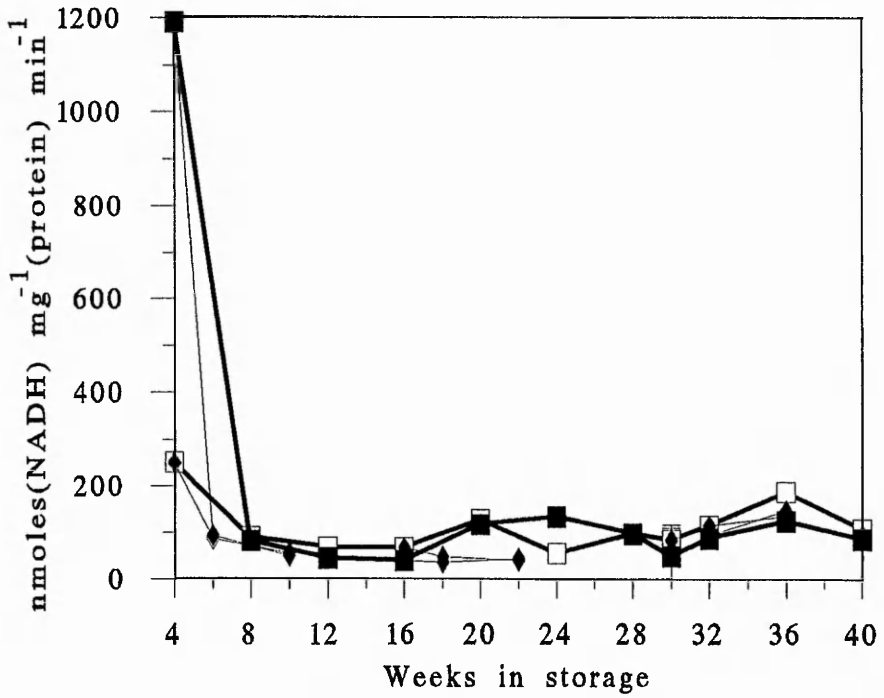


Figure 3.37. The NADH-GOGAT activity of tissue from Pentland Dell tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1991-92 storage season.

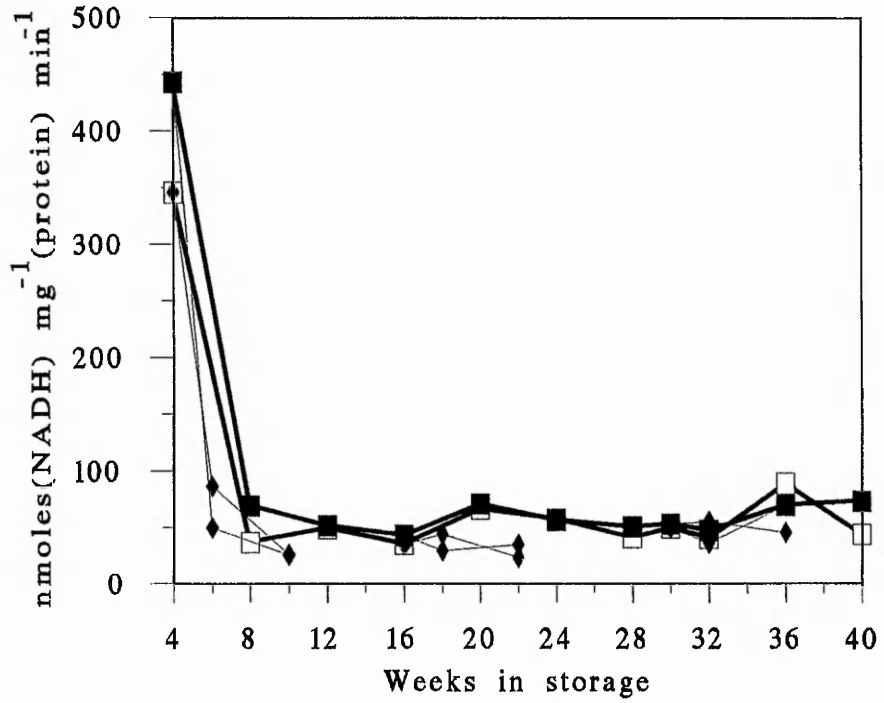


Figure 3.38. The NADH-GOGAT activity of tissue from Record tubers stored at 5°C(□), 10°C(■) and reconditioned at 20°C(◆), 1991-92 storage season.

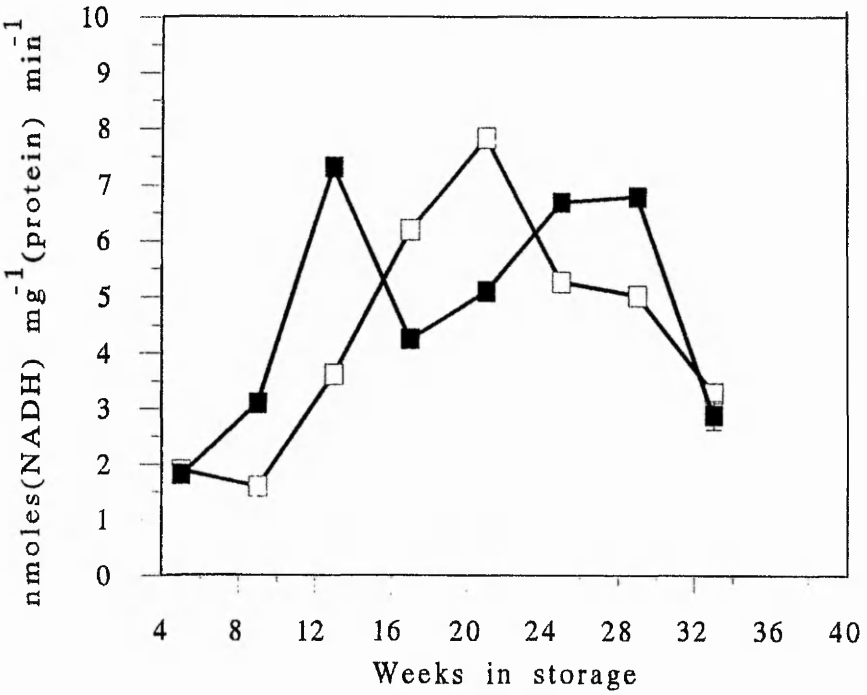


Figure 3.39. The NADH-GOGAT activity of tissue from Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

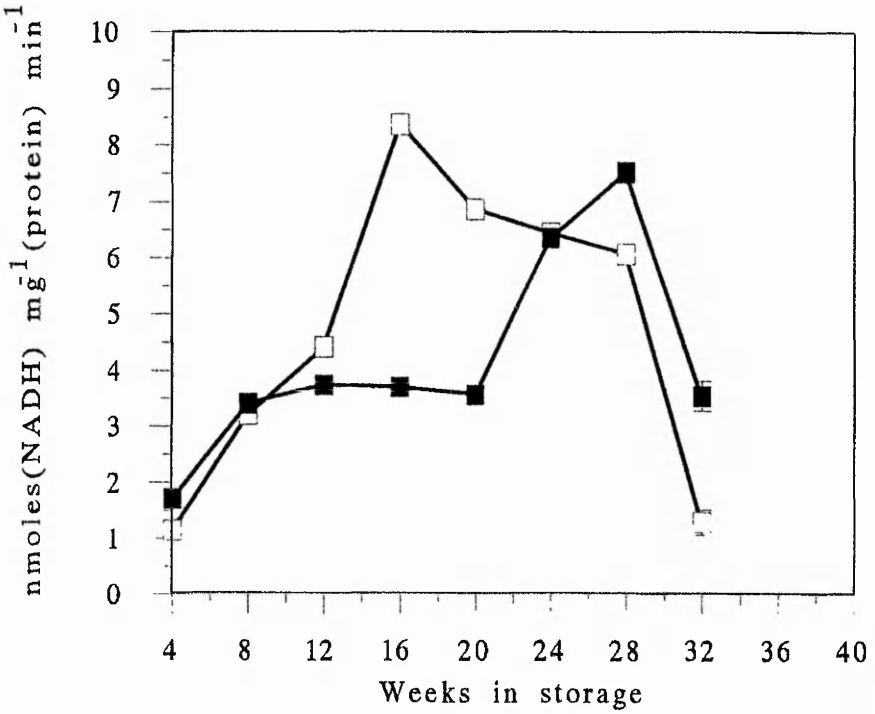


Figure 3.40. The NADH-GOGAT activity of tissue from Record tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

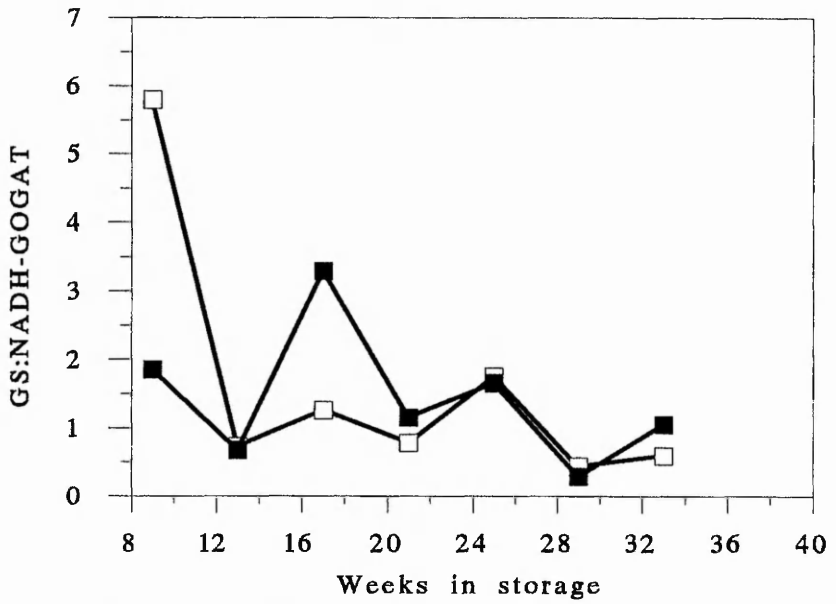


Figure 3.41. The ratio of glutamine synthetase to NADH-GOGAT activity in Pentland Dell tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

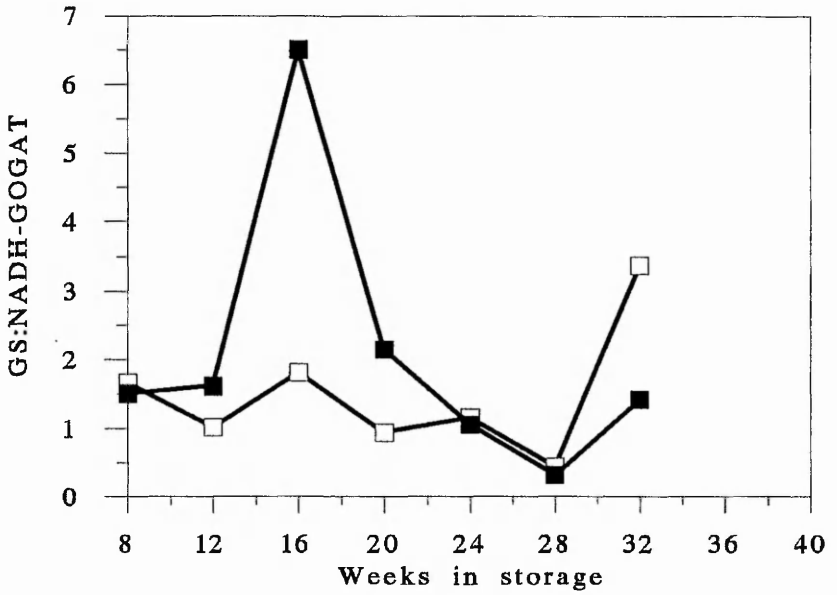


Figure 3.42. The ratio of glutamine synthetase to NADH-GOGAT activity in Record tubers stored at 5°C(□) and 10°C(■), 1992-93 storage season.

3.4. DISCUSSION

An examination of the literature has suggested that both proteolysis and protein synthesis may occur during prolonged storage of potato tubers, the net direction of nitrogen flow depending on their dormancy state (Levitt, 1954; Cotrufo and Levitt, 1958; Ashford and Levitt, 1965; Nowak, 1977). In this study, an early season increase (up to 11 weeks) of soluble protein was exhibited by both Pentland Dell and Record in 1989-90 and 1991-92 (Figs. 3.02., 3.04., 3.06. and 3.08.). This trend implied that some protein synthesis occurred over the first 3 to 4 months of storage when the tubers were dormant (Levitt, 1954), higher storage temperatures accelerating this accumulation of soluble protein in Pentland Dell. The growth of the soluble protein pool was not linked to a drop in free amino acids with the exception of Pentland Dell stored at 10°C in 1991-92 (Figs. 2.07., 2.09., 2.11. and 2.13). This trend may imply that a degree of amino acid translocation takes place from external to internal tissues, in order to provide for protein synthesis (Fig. 3.43.). Such a translocation was proposed for dormant Russet tubers between November and January by Cotrufo and Levitt (1958). A similar effect may take place throughout the majority of the 1992-93 season in both cultivars and also over the latter half of 1991-92 in Pentland Dell and 1990-91 in Record (Figs. 3.04. and 3.07.). In Pentland Dell in the 1991-92 and 1992-93 storage seasons, both the increase of soluble protein as a result of probable protein synthesis (Levitt, 1954; Cotrufo and Levitt, 1958; Nowak, 1977) and the rise of free amino acids (Figs. 2.08. and 2.09.) demonstrated higher rates at 10°C than 5°C, this implying a greater rate of amino acid transport at the higher storage temperature.

The studies of Cortrufo and Levitt (1958), Ashford and Levitt (1965) and Nowak (1977) showed protein degradation after harvest followed by subsequent synthesis until the break of tuber dormancy. Upon emergence from the rest period the net flow of nitrogen was reversed, with protein degradation providing a source of free amino acids to support sprout growth. In this study, a similar trend was observed for Record

in 1990-91 (Fig. 3.07.). The drop in soluble protein over the first 16 weeks of 1990-91 in both Pentland Dell and Record (Fig. 3.07.) was not accompanied by a marked increase of free amino acids, possibly suggesting an outward translocation of free amino acids from the tuber core. Such a movement was postulated by Cortrufo and Levitt (1958), their results indicating this direction of translocation from harvest until early November.

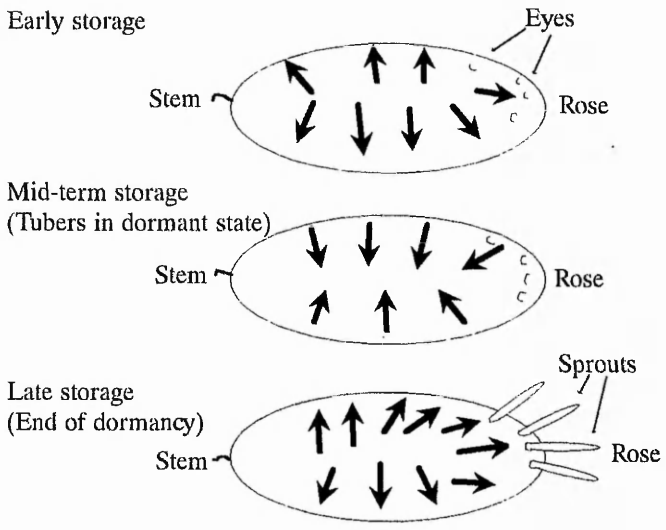


Figure 3.43. Translocation of free amino acids between internal and external tuber tissues during prolonged storage.

In Pentland Dell (1990-91), the flow of nitrogen into the amides upon protein degradation implied their probable importance as key nitrogen transport compounds both in the tuber and the whole plant on sprouting (Figs. 2.07., 2.16 and 2.20.). A similar trend also occurred at the beginning of 1992-93 in both cultivars and after 28 weeks in Record in the 1990-91 season, in the latter case, the soluble protein pool displaying a decline until the end of storage. It is likely that this late season decline of protein (Levitt, 1952; Levitt, 1954; Levitt and Cotrufo, 1958; Nowak, 1977; Davies and Ross, 1984) is a result of proteolysis (Nowak and Skwiercz, 1975; Davies and Ross, 1987), the resultant free amino acids being transported to the external tissues (Fig. 3.43.) in order to support sprout growth (Davies and Ross, 1987). The studies

of Davies and Ross (1984) suggested that protein degradation associated with sprouting first occurred in the perimedulla tissue containing the internal phloem, this further supporting the above translocation hypothesis.

The proteolytic enzyme activity extracted from Pentland Dell and Record in the 1992-93 storage season displayed a high rate at 4 to 5 weeks of storage (Figs. 3.29. and 3.30.), this with the low soluble protein content (Figs. 3.05. and 3.09.) further suggesting the net degradation of proteins during early storage (Cotrufo and Levitt, 1958; Ashford and Levitt, 1965; Nowak, 1977). The minimal rate of proteolytic activity from 8-9 to 20-21 weeks was consistent with the observations of Nowak and Skwiercz (1975), this earlier study showing low activity in the parenchyma and eyes of stored Baca and Bem tubers during the rest period. In this study, this minimal rate of proteolysis was concomitant with the rise in soluble protein content (Figs. 3.05., 3.09., 3.29. and 3.30.). The net flow of nitrogen was hence in the direction of protein synthesis (Cotrufo and Levitt, 1958; Ashford and Levitt, 1965; Nowak, 1977), the temperature dependence of this synthesis (Nowak, 1977) being reflected by the soluble protein pool size in Pentland Dell (Fig. 3.05.). This trend of low proteolytic activity and the resultant direction of net nitrogen flux, suggested that Pentland Dell and Record were dormant during this period (Cotrufo and Levitt, 1958; Nowak and Skwiercz, 1975; Nowak, 1977).

After 20-21 weeks storage, the proteolytic enzyme activity of Pentland Dell and Record increased dramatically, hydrolysing protein to produce a marked upturn of free amino acids, in particular the amides (Figs. 3.29. to 3.32. and Table 3.2.). This rise of proteinase activity may result from *de novo* synthesis of the enzyme, as occurs on the ending of dormancy in a variety of seeds (Chrispeels, Baumgartner and Harris, 1976). In this study, the temperature dependence of proteolytic activity in Pentland Dell was concurrent with the greater increase of total free amino acids and amides observed at 10° than 5°C (Figs. 3.29. to 3.32.). This higher rate of enzyme activity at

10°C was inconsistent with the findings of Nowak and Skwiercz (1975), these suggesting low temperature enhancement of protein degradation. The mobilisation of nitrogen as a result of this increase in proteolysis suggested that Pentland Dell and Record were breaking dormancy at this point in storage (Levitt, 1952; Levitt, 1954; Cotrufo and Levitt, 1958; Ashford and Levitt, 1965; Nowak and Skwiercz, 1975; Nowak, 1977). However, the synthesis of protein over the latter half of the 1992-93 season suggested there was little flow of nitrogen into the forming sprouts. Davies and Ross (1984) postulated a source-sink relationship of nitrogen flux between tubers and sprouts as has been demonstrated for reserve mobilisation in various dicotyledonous seeds (Davies and Slack, 1981). It has also been proposed that gibberellic acid may play a role in the regulation of tuber reserve mobilisation (Bailey, Phillips and Pitt, 1978). As the enzymes of starch degradation were unaffected by gibberellic acid treatment, any control of mobilisation is probably through effects on the cell compartmentation of the enzymes of reserve hydrolysis (Bailey *et al*, 1978). In this study, a relative lack of sprout growth resulting from CIPC treatment of stored tubers suggested a low sink strength, as a result it is probable that nitrogen mobilisation never reaches it's maximum potential in sprout inhibited tubers.

In addition to the hydrolysis of soluble protein reserves, degradation of insoluble proteins may also play a role in the mid to late season accumulation of free amino acids. However, the results of Racusen (1983) suggested that such a decrease of insoluble protein does not occur in storage, the soluble protein fraction alone displaying degradation. The insoluble protein fraction was shown by Neuberger and Sanger (1942) to comprise between 8% and 12.5% of the total tuber nitrogen in a number of varities, this being concentrated in the outer layers of tuber tissue, in particular the skin (Cook, 1921 cited by Neuberger and Sanger, 1942; Neuberger and Sanger, 1942).

Increases in the free amino acid content observed for Pentland Dell tubers in the 1989-90, 1991-92 and 1992-93 seasons (Figs. 2.06., 2.08. and 2.09.) and Record tubers in all seasons (Figs. 2.10. to 2.13.) were not always consistent with a decrease in soluble protein. It is therefore likely that proteolysis and protein synthesis may occur in the same period of storage. This implies the occurrence of protein turnover, with the net direction of nitrogen flow depending on both the activity of proteolytic enzymes and the degree of protein synthesis. The results of Nowak (1977) support such a turnover of protein during storage, the high molecular weight tuber protein fraction decreasing during the rest period, whereas low molecular weight proteins were observed to increase. Low storage temperatures were observed to lessen the synthesis of these low molecular weight proteins (Nowak, 1977), this providing a possible explanation for the effect of storage temperature on the protein increases in Pentland Dell observed in this study (Figs. 3.04., 3.05. and 3.07.). This temperature influence on protein synthesis together with that noted for proteolysis in Pentland Dell (Fig. 3.29.) implied a higher rate of protein turnover at 10° than 5°C.

Reconditioning treatments produced inconsistent effects on soluble protein content (Figs. 3.01. to 3.03. and 3.05. to 3.07.), due to an ineffectiveness in controlling the free amino acid pool size (Figs. 2.06. to 2.08. and 2.10. to 2.12.). It is unlikely that these treatments alter the direction of net nitrogen flow relative to that of non-reconditioned tubers at any given time. The higher soluble protein pool size observed in late season reconditioned Pentland Dell and Record (transferred from 10° to 20°C) relative to that of those at 10°C (1990-91 storage season) was matched by a higher free amino acid content (Figs. 2.07., 2.11., 3.03. and 3.07.). This suggested that protein degradation and synthesis (Nowak, 1977) were equally affected by the higher temperature, with the rate of protein turnover increasing.

The soluble protein pool composition observed in this study was generally consistent with the results of Racusen and Foote (1980), Paiva, Lister and Park (1982),

Gorenstein, Yamagata and Hadziyev (1988), Hannapel (1990) and Suh, Peterson, Stiekema and Hannapel (1990), with patatin (44-45kDa) and the 20-25kDa protein fraction comprising a major proportion of the soluble protein pool (Fig. 3.18.). The study of protein pool composition by gel-filtration chromatography showed the first protein fraction eluted to have an approximate molecular weight of 88kDa, this protein displaying a monomeric molecular weight of 44-45kDa when analysed by SDS-PAGE (Fig. 3.19.). These results together with the observed lipid acyl hydrolase activity concurred with the properties of the glycoprotein patatin shown by the studies of Racusen and Foote (1980), Park, Blackwood, Mignery, Hermodson and Lister (1983), Racusen (1983), Racusen and Weller (1984), Racusen (1985), Racusen (1986), Liedl, Kosier and Desborough (1987) and Andrews, Beames, Summers and Park (1988). The second gel filtration chromatography fraction eluted consisted of three bands between 20 and 25kDa and a further 17-18kDa band (Fig. 3.19.), suggesting this fraction to contain the 22kDa protein complex observed by Hannapel (1990) and Suh *et al* (1990). The complex was observed to contain 3 immunologically related proteins with molecular weights of 22, 23 and 24kDa (Suh *et al*, 1990). This fraction also demonstrated the ability to inhibit trypsin, in agreement with the findings of Hannapel, Stiekema and Suh (1991) which showed the 22 and 23kDa proteins to inhibit trypsin and chymotrypsin, and the 24kDa protein to demonstrate inhibition against trypsin only.

Patatin comprised a similar proportion of the soluble protein pool (Figs. 3.24., 3.25., 3.26. and 3.27.) as that observed by Racusen and Foote (1980), Park *et al* (1983) and Racusen (1985), this generally being between 12 and 30%. Slight variations were observed between Pentland Dell and Record, patatin comprising a higher proportion of the soluble protein content in the former, this cultivar variation being consistent with the differences between Kennebec and Desiree observed by Racusen (1985). Patatin content was also observed to decline during the prolonged storage of Kennebec at 4°C by Racusen (1983). The proportion of the soluble protein pool

consisting of this glycoprotein declined over the latter half of storage. In this study, patatin declined relative to the soluble protein pool in the 1991-92 (In Pentland Dell 14% at 5°C, 43% at 10°C and in Record 32% and 48% respectively) and 1992-93 (In Pentland Dell 37% at 5°C, 51% at 10°C and in Record 32% and 49% respectively) seasons (Figs. 3.24. to 3.27.). The temperature dependent decrease of patatin pool size inferred that hydrolysis of the glycoprotein is greater in 10°C stored tubers. The free amino acids produced by this degradation (Figs. 3.20. to 3.23.) accounted for only a fraction of the free amino acid accumulation observed during storage (Figs. 2.08., 2.09., 2.12. and 2.13.). It is thus possible that patatin plays a relatively minor role as a storage protein, patatin's function as an enzyme (Racusen, 1985; Racusen, 1986; Andrews *et al*, 1988; Davis, Currier and Racusen, 1989) likely being of more physiological significance. The breakdown of patatin during storage, coupled to the increase of total soluble protein observed in the 1992-93 season (Figs. 3.05. and 3.09.) is further evidence that protein turnover occurs, the free amino acids from patatin hydrolysis possibly forming a proportion of those supplying the synthesis of new proteins. The temperature dependent nature of patatin degradation in conjunction with that of the increase in soluble protein, further inferred a greater rate of protein turnover at higher storage temperatures.

The marked substrate specificity of the extracted proteolytic enzyme activity for endogenous tuber proteins (Table 3.3.) confirmed that the activity exhibited was of physiological significance to tuber nitrogen metabolism. The substrate specificity demonstrated against patatin (Table 3.3.) inferred that the degradation of this glycoprotein is at least in part accounted for by this observed proteolytic activity. The high rates of proteolysis displayed by both cultivars were generally coincident with decreases of patatin content (Figs. 3.21., 3.23., 3.29. and 3.30.), the temperature dependence of the mid to late season increase of proteolysis in Pentland Dell being reflected by the patatin pool decline.

As with patatin, the 22kDa protein complex and various other tuber proteins contain a high proportion of aspartate, glutamate, asparagine and glutamine residues (Racusen and Foote, 1980; Liedl *et al*, 1987; Gorinstein *et al*, 1988; Suh *et al*, 1990), it is likely that a large proportion of these amino acids were present in the tuber free amino acid pool as a direct result of protein hydrolysis. Although the tuber amide content correlated closely with proteolytic enzyme activity in the 1992-93 storage season (Table 3.1.), the proportion of amide residues present in the major tuber proteins was not high enough to account for the entire increase of amides observed (Figs. 3.31. and 3.32.). A degree of amino acid amidation subsequent to protein degradation was hence likely (Fig. 3.44.), this being supported by the observed activity of glutamine synthetase in Pentland Dell and Record over the 1992-93 storage season (Figs. 3.34. and 3.35.). Both cultivars also displayed considerable activity of NADH-GOGAT, this being the key enzyme of glutamine deamidation (Fig. 3.44.) in non-green plant tissues such as roots, seeds and tubers (Beevers and Storey, 1976; Dilworth and Dure, 1978; Salisbury and Ross, 1978; Osuji, Cuero and Washington, 1991).

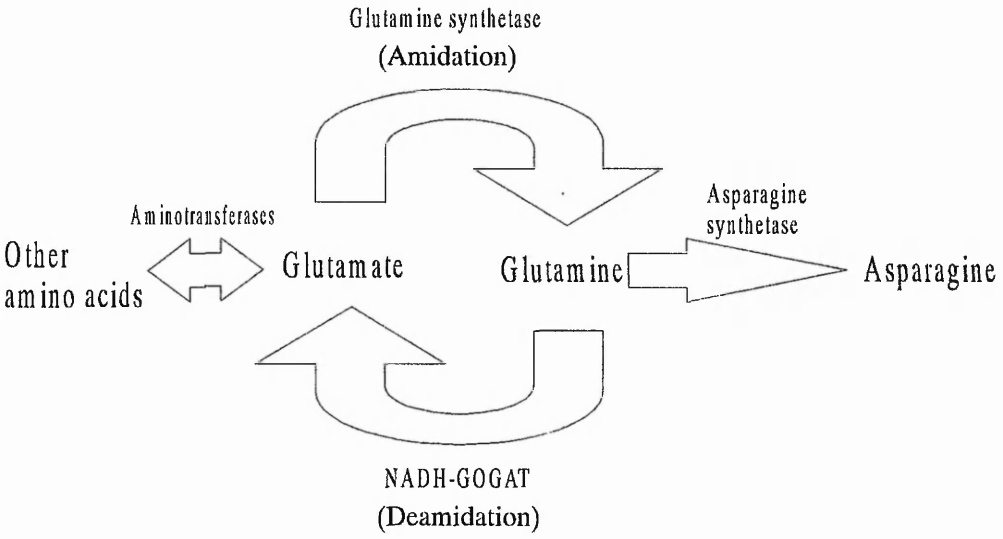


Figure 3.44. A simple model for the amidation and deamidation of free amino acids during tuber storage.

The activities of these enzymes exhibited by Pentland Dell and Record tuber tissue in the 1992-93 storage storage season (Figs. 3.34., 3.35., 3.39., 3.40.,3.41. and 3.42.) suggested a potential net amidation of amino acids over the majority of the season, this accounting for the high proportion of glutamine and asparagine in the free amino acid pool. This trend was consistent with the increase of amides at 24-25 weeks (Figs. 3.31.,3.32., 3.41. and 3.42.), the high rate of protein degradation supplying free amino acids for conversion into glutamine by the activity of glutamine synthetase, and subsequently asparagine through the probable activity of asparagine synthetase. The trend of free amino acid amidation displayed no consistent influence of temperature. This implies that the temperature dependence of amide content in Pentland Dell (1992-93 storage season) is solely a result of greater proteolytic activity at 10° than 5°C (Fig. 3.31). As both glutamine and asparagine increased equally during storage (1992-93 storage season), it is likely that the activities of both glutamine synthetase and asparagine synthetase were present throughout storage. These results suggest that both amides played an equally important role in the transport of nitrogen within the tuber, this overall process of nitrogen mobilisation after the break of dormancy displaying some similarity with that observed in a variety of seeds upon germination (Streeter, 1973; Capdevilla and Dure, 1977; Dilworth and Dure, 1978).

The results in this Chapter have indicated the key importance of proteolytic enzyme activity in determining the size of the free amino acid pool during tuber storage. The activity studied throughout tuber storage required further characterisation in order to determine the types of enzyme involved. In the following Chapter the mechanistic class of this proteolytic activity was examined by the use of class specific inhibitors. The key class of enzyme identified was further purified in order to ascertain the number of enzymes involved.

3.5. CONCLUSIONS

The following conclusions may be deduced from the results detailed in this Chapter.

Firstly, both protein synthesis and degradation occur during prolonged tuber storage, the rate of this protein turnover being greater at higher storage temperatures and as a result of reconditioning treatments. In cases where both free amino acid and soluble protein pools increased simultaneously, the translocation of additional free amino acids from external tissues to the tuber core may be implicated to supply the protein synthesis.

Secondly, the direction of net nitrogen flow between proteins and free amino acids depended on the state of dormancy of the tuber, this varying between storage season and cultivar. In general, tubers exhibited a trend of net protein degradation attributable to high proteolytic enzyme activity on entry into storage. When tubers underwent dormancy this nitrogen flux reversed until the end of the rest period with pronounced protein synthesis occurring in the tuber core tissues in addition to a minimal rate of protein degradation. The break of tuber dormancy was characterised by a marked switch back to net protein degradation, increased proteolytic enzyme activity mobilising tuber core nitrogen reserves in order to supply sprout growth. Decreases of protein content were not always mirrored by increases of free amino acids possibly due to the transport of amino acids from the tuber core to the external tissues.

Thirdly, the overall direction of nitrogen flux was independent of storage temperature, Pentland Dell tubers demonstrating an increased rate of both protein synthesis and degradation at the higher storage temperature. However, when net nitrogen flow exhibited a distinct direction this was generally enhanced by increased temperatures.

Reconditioning treatments were hence predominately ineffective as a means of controlling the free amino acid content of tuber tissue.

Fourthly, patatin and the 22kDa protein complex were the most abundant soluble tuber proteins, patatin alone comprising between 12% and 30% of the soluble protein pool. Both patatin and the 22kDa complex were acted on by tuber proteolytic enzymes with the decrease of patatin accounting for only a fraction of the increase in free amino acids with storage duration.

Finally, the amino acid composition of the major tuber proteins could only partially account for the high proportion of amides present in the free amino acid pool. Free amino acids produced by protein degradation underwent amidation to glutamine through the action of glutamine synthetase. Subsequent asparagine formation was probably performed by asparagine synthetase, asparagine exhibiting no increase relative to glutamine due to the continued activity of glutamine synthetase throughout storage. Activity of NADH-GOGAT was generally insufficient to stop the net amidation of free amino acids. This trend of net amidation was independent of storage temperature, the higher amide content in 10°C stored Pentland Dell tubers relative to those at 5°C was hence due to greater proteolytic enzyme activity.

CHAPTER 4. CHARACTERISATION OF PROTEOLYTIC ENZYME ACTIVITY FROM STORED TUBERS

4.1. INTRODUCTION

Studies on leaves, fruit, seeds and tubers from a wide variety of plants have shown several classes of proteolytic enzyme to be involved in protein turnover and storage protein mobilisation (Ryan, 1973; Wilson, 1986). A proteolytic enzyme may be classified according to both the catalytic mechanism and specificity of activity. Four groups of protease have been identified in terms of catalytic mechanism, these are the cysteine proteases, serine proteases, metalloproteases and aspartic proteases (Hartley, 1960). The catalytic specificity of proteolytic enzymes has been divided into two main forms, the proteinases and peptidases. Proteinases operate by hydrolysing the internal peptide bonds of proteins hence producing peptides as the products, whereas peptidases cleave the peptide bond of either the carboxyl or amino terminal amino acid residue, the resultant products consisting of at least one free amino acid. The sub-classes of peptidases are known as carboxypeptidases and aminopeptidases respectively, depending on the terminal peptide bond that is hydrolysed.

Studies on the mobilisation of protein reserves in germinating seeds have predominately shown proteinases and carboxypeptidases with acidic pH optima to be involved, associated with protein bodies that contain the bulk of the seed storage proteins (Yatsu and Jacks, 1968; St Angelo, Ory and Hansen, 1969; St Angelo, Ory and Hansen, 1970; Harris and Chrispeels, 1975; Baumgartner, Tokuyasu and Chrispeels, 1978; Nishimura and Beevers, 1978; Tully and Beevers, 1978; Van der Wilden, Herman and Chrispeels, 1980; Yu and Greenwood, 1994). Although the potato tuber storage proteins such as patatin and the 22kDa complex are probably located in the vacuole (Sonnewald, Sturm, Chrispeels and Willmitzer, 1989; Sonnewald, Von Schaewen and Willmitzer, 1990; Suh *et al*, 1990) rather than in

protein bodies as in the cotyledons of seeds, it is possible that similar types of enzymes are involved in their degradation. Proteinase, carboxypeptidase and aminopeptidase activities have been extracted from potato tubers (Hojima, Tanaka, Moriya and Moriwaki, 1971; Nowak and Skwiercz, 1975; Santarius and Belitz, 1978; Davies and Ross, 1986). The carboxypeptidase and aminopeptidase probably utilise the serine protease and cysteine protease catalytic mechanisms respectively (Santarius and Belitz, 1978).

In this study, the proteolytic enzyme activity (proteinase) observed throughout the 1992-93 storage season (Chapter 3) has been characterised, the aim being to ascertain the classes and numbers of enzymes comprising this activity. A crude classification of tuber proteinases by catalytic mechanism was performed by the use of class specific inhibitors. The key class of activity distinguished by this study was further purified by the use of anion exchange, affinity and gel-filtration chromatography. The pure proteinase was separated by SDS-PAGE in order to determine the monomeric molecular weight.

4.2. MATERIALS AND METHODS

4.2.1. Determination of proteinase mechanistic class.

Tuber proteinase activity was classified into mechanistic group by the use of protease inhibitors specific for a particular class of enzyme. Proteinase activity was partially purified (as described in Chapter 3) from Pentland Dell stored for 18 weeks at 5°C. The extract was pre-incubated with the respective inhibitors in microtitre plate wells prior to the addition of substrate and activity compared with control wells containing no inhibitor.

The following inhibitors were made up as stock solutions (Table 4.1.)

Table 4.1. Protease inhibitors and their target enzymes.

Inhibitor	Supplier	Target enzymes	Stock concentration
PMSF (phenylmethyl-sulphonyl fluoride)	Sigma	serine proteases	200mM in methanol
pepstatin A	Sigma	aspartic proteases	1mM in DMSO
1,10-phenanthroline	Sigma	metalloproteases, metal activated proteases	200mM in methanol
E64	Sigma	cysteine proteases	250μM in distilled water
EDTA dihydrate disodium salt	Sigma	metalloproteases, metal activated proteases	100mM in assay buffer
cysteine	Sigma	reducing agent sensitive proteases	100mM in assay buffer

The inhibitor stock solutions were diluted to within their effective concentration ranges (Santarius and Belitz, 1978) with assay buffer, 100mM citric acid (Sigma) - disodium hydrogen phosphate (AnalaR, BDH) pH 6.0 (McIlvaine, 1921 cited by

Dawson *et al*, 1969) containing 10mM NaCl. Each inhibitor was used at 3 concentrations within this range (Table 4.2.).

Table 4.2. Protease inhibitor concentration in assay buffer.

Inhibitor	Concentration
PMSF	20mM, 2mM and 200μM
pepstatin A	100μM, 10μM and 1μM
1,10-phenanthroline	20mM, 2mM and 200μM
E64	100μM, 10μM and 1μM
EDTA	100mM, 10mM and 1mM
cysteine	100mM, 10mM and 1mM

Prior to the addition of substrate, a 20μl inhibitor aliquot was added to 20μl enzyme extract in a microtitre plate well and incubated at 30°C for 30 min. Following this pre-incubation, 20μl of FITC-casein substrate (Produced as described in Chapter 3; Twining, 1984) was added and the reaction mixture incubated at 30°C for a further 2 hours. The reaction was subsequently terminated by the addition of 120μl 5%(w/v) TCA (AnalaR, BDH). Each treatment was compared with wells in which the reaction had not proceeded due to the addition of TCA prior to the addition of the substrate.

Following termination, the microtitre plate was allowed to stand for 1 hour at room temperature. The well contents were transferred by Pasteur pipette into microfuge tubes and the precipitated protein sedimented for 5 min using an MSE Micro-Centaur microfuge on the high speed setting. Following centrifugation, 60μl aliquots of supernatant were diluted to 3ml with 500mM Tris-HCl pH 8.5 and gently mixed to ensure the entire sample was at the correct pH. The fluorescence of the samples was determined as described in Chapter 3 and the protein content of the enzyme extract

measured by Coomassie Blue dye binding (Bradford, 1976). Proteinase specific activity was expressed as relative fluorescence units mg^{-1}(protein) min^{-1}, the degree of inhibition being the percentage inhibition relative to the respective control samples which contained no inhibitor in the reaction mixture (assay buffer was substituted for inhibitor in these controls).

The above study was repeated at a more comprehensive range of concentrations (100μM, 10μM, 1μM, 100nM, 10nM, 1nM and control *ie.* no inhibitor) for the aspartic protease inhibitor pepstatin A (Sigma), in order to obtain an inhibition curve. As the 1mM pepstatin A stock solution was made up in DMSO (Analytical Reagent, Fisons), the effect of this solvent alone on proteinase activity was also examined. A 1% (v/v) DMSO treatment in assay buffer was pre-incubated with enzyme extract as for the inhibitor solutions. The assay was carried out as for the above study and the degree of pepstatin A inhibition expressed as percentage inhibition relative to the uninhibited control sample.

4.2.2. Purification of aspartic proteinase activity.

Aspartic proteinase activity was purified from Pentland Dell tubers stored for 36 weeks at 10°C. Two kg of whole peeled tubers was homogenised in a chilled Waring blender with 100mM citric acid-disodium hydrogen phosphate buffer pH 6.0 as described above (McIlvaine, 1921 cited by Dawson *et al*, 1969), additionally containing 2mM EDTA (Sigma, dihydrate disodium salt), 5mM cysteine (Sigma) and 10mM NaCl (Sigma). This homogenate was filtered through 2 layers of muslin and then clarified in an MSE 18 refrigerated centrifuge at 4000g for 30 min. The resulting supernatant had a volume of 720ml and was used for $(NH_4)_2SO_4$ precipitation at 10°C. Solid $(NH_4)_2SO_4$ (AnalaR, BDH) was added gradually with gentle stirring to the extract until 90% saturation was achieved. The extract was then spun at 6000g for 1 hour at 5°C to sediment the precipitated protein. The protein was then resuspended in 80ml extraction buffer and dialysed for 24 hours against 5L of 0.25M Tris-HCl pH

7.2 containing: 2mM EDTA dihydrate disodium salt (Sigma) and 2.5mM 2-mercaptoethanol (Sigma). A 2ml sample of the dialysed extract was frozen in liquid N_2, stored at -70°C and used to determine the protein content by Coomassie Blue dye binding (Bradford, 1976) and protein composition by SDS-PAGE (Laemmli, 1970). The proteinase activity of this dialysed protein extract was determined as described in Chapter 3 (Twining, 1984).

The remaining 78ml of dialysed extract was further purified by anion exchange chromatography using a 50ml Fast Flow Q-Sepharose (Pharmacia) column and Bio-Rad Econo Preparative Liquid Chromatography system. This consisted of an Econo System controller, pump, uv-monitor, model 2110 fraction collector and model 1326 chart recorder. The sample was pumped onto the column at a flow rate of 2ml min^{-1}. Once all the sample had entered the column, unbound protein was removed by running start buffer (0.25M Tris-HCl pH 7.2 containing 2mM EDTA dihydrate disodium salt and 2.5mM 2-mercaptoethanol; both from Sigma) through the column at a flow rate of 2ml min^{-1} and absorbance of the eluant was monitored at 280nm. After all the unbound protein had eluted, the proteins bound to the anion exchanger because of their overall negative charge at pH 7.2, were separated by running a 0-2M NaCl gradient.

Bound tuber proteins were eluted by running a NaCl gradient that increased from 0 to 2M over 1 hour. The above start buffer was used as the initial mobile phase at a flow rate of 2ml min^{-1}, whilst an identical buffer with the addition of 2M NaCl (AnalaR, BDH) was used to create the salt gradient. Protein elution was monitored by recording the absorbance of the eluant at 280nm and 1ml fractions collected from the start of the gradient.

Every fifth protein-containing fraction was assayed for proteinase activity using the method described previously (Twining, 1984) and the active fractions pooled. A 2ml

sample of these pooled active fractions was frozen in liquid N_2, stored and used to determine pooled proteinase activity, protein composition by SDS-PAGE (Laemmli, 1970) and protein content by Coomassie Blue dye binding (Bradford, 1976).

The pooled fractions were further purified by affinity chromatography using a 2ml column packed with pepstatin-agarose (Sigma). The column was equilibrated with start buffer; 100mM citric acid-disodium hydrogen phosphate pH 6.0 (McIlvaine, 1921 cited by Dawson *et al*, 1969) containing 500mM NaCl (AnalaR, BDH), 5mM cysteine (Sigma) and 2mM EDTA dihydrate disodium salt (Sigma). The enzyme extract was run onto the column at a flow rate of 1ml min^{-1} and the absorbance monitored continuously at 280nm. Further start buffer was run through the column until the absorbance at 280nm was zero. The inhibitory property of pepstatin-A was utilised in binding aspartic proteinase in the tuber extract to the stationary phase. To reverse this specific binding and elute the proteinase by reversibly inactivating the enzyme, the pH was reduced by running glycine-HCl pH 2.2 elution buffer through the column. This buffer was made up of 250ml 0.2M glycine (Sigma) and 220ml 0.2M HCl (diluted from 1M AnalaR, BDH) diluted to 1L with distilled water. The elution was carried out at a 1ml min^{-1} flow rate, monitored at 280nm and 1ml fractions collected.

The pH of the collected fractions was increased to pH 6.0 by the addition of 10 drops 500mM citric acid-disodium hydrogen phosphate buffer (McIlvaine, 1921 cited by Dawson *et al*, 1969) reversing the pH inactivation of the proteinase extract. Every second fraction was assayed for proteinase activity as described earlier (Twining, 1984) and the active fractions pooled. A 2ml sample of the pooled fractions was frozen in liquid N_2 and stored, for the determination of specific activity, protein content (Bradford, 1976) and protein composition (Laemmli, 1970).

The pooled proteinase fractions were put through a final purification step using a 250ml column packed with the gel-filtration medium Sephacryl S-300HR (Pharmacia). The column was equilibrated with proteinase assay buffer (100mM citric acid-disodium hydrogen phosphate pH 6.0; McIlvaine, 1921 cited by Dawson *et al*, 1969) at a flow rate of 1ml min^{-1} and calibrated using protein molecular weight standards. A 10mg ml^{-1} solution of blue dextran (Sigma) was diluted to 50% (v/v) with assay buffer and 2ml injected onto the column via the sample fill loop. The elution at 1ml min^{-1} with assay buffer was monitored at 280nm, with the elution volume of blue dextran (2000kD) being equivalent to the column void volume (Vo). The elution volumes (Ve) of the following individual 5mg ml^{-1} protein standards (Sigma) were also determined, β–amylase (200kDa), alcohol dehydrogenase (150kDa), bovine serum albumin (66kDa), carbonic anhydrase (29kDa) and cytochrome C (12.4 kDa). In each case 2ml was injected onto the column. A calibration curve for molecular weight was prepared by plotting log molecular weight against Kav, where

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

Vt = the total accessible column volume, Ve = the elution volume of the protein and Vo = the column void volume, this enabling the molecular weight of the proteins in the proteinase extract to be calculated. The enzyme extract (2ml) was loaded onto the column and subsequently eluted at 1ml min^{-1} with the above assay buffer. The absorbance of the eluant was monitored at 280nm and 5ml fractions collected after the void volume of the column had eluted. The fractions comprising each eluted protein peak were assayed for proteolytic enzyme activity (Twining, 1984). The fractions from the active proteinase peak were pooled and frozen in liquid N_2 for the determination of specific activity (Twining, 1984), protein content (Bradford, 1976) and monomeric molecular weight by SDS-PAGE (Laemmli, 1970). The elution volume of the proteinase peak (Ve) was used to estimate the native molecular weight from the gel-filtration calibration curve.

4.3. RESULTS

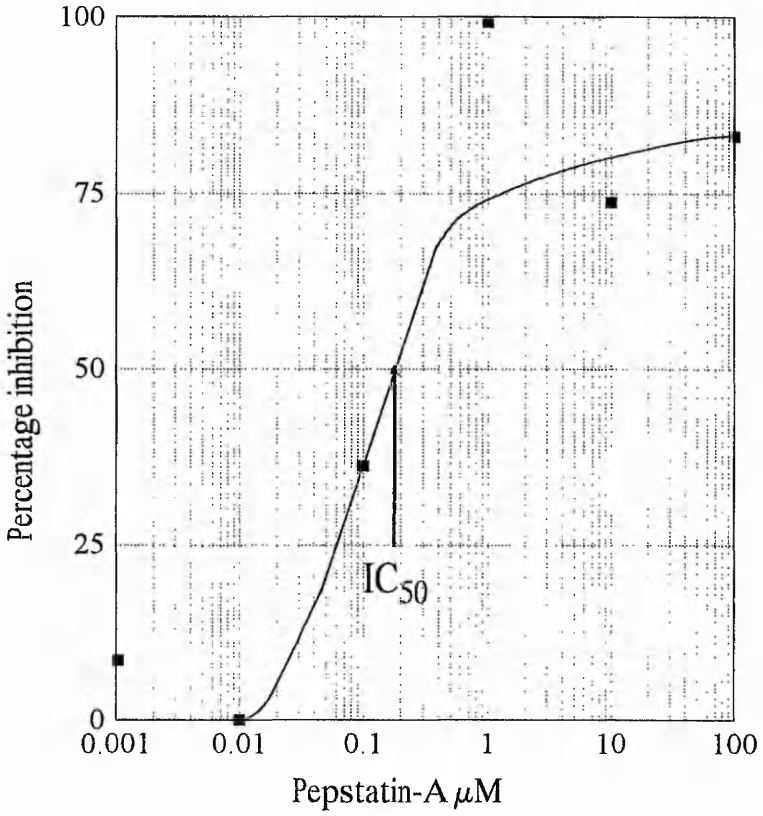
4.3.1. The catalytic mechanism of tuber proteinase activity.

Tuber proteinase activity was inhibited up to 25% by the serine protease inhibitor, PMSF, this suggesting only minimal activity of this class of enzyme during mid-term storage at 5°C of Pentland Dell (Table 4.3.). In contrast, the aspartic protease inhibitor, pepstatin-A, caused almost total inhibition of the observed proteinase activity (Table 4.3.). In addition, the inhibitor of metalloproteases and metal activated proteases, 1,10-phenanthroline, brought about a 45% inhibition of tuber proteinase activity, the greatest degree of inhibition occurring at the highest inhibitor concentration (20mM). The metal chelator, EDTA produced a similar degree of inhibition at concentrations of 1mM and 10mM (Table 4.3.), however at a concentration of 100mM no inhibitory effect of EDTA was observed. The cysteine protease inhibitor, E64 (L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane) had no effect on proteinase activity (Table 4.3), whilst cysteine an inhibitor of reducing agent sensitive proteases (Santarius and Belitz, 1978), produced 43% inhibition at a 1mM concentration. This effect was markedly reduced at 10mM and a 100mM concentration brought about over 100% stimulation of proteinase activity (Table 4.3.).

A more detailed dose-response study of the inhibition of tuber proteinase activity by pepstatin-A, showed a concentration of 0.15μM inhibitor to be the IC_{50} value, this bringing about a reduction in activity of 50% for an enzyme concentration of 1 mg (protein) ml^{-1} assay mixture (Fig. 4.01.). A pepstatin-A concentration of 1μM completely inhibited activity in an assay mixture containing the above enzyme concentration. Inclusion of 1% (v/v) DMSO in an assay mixture in the absence of pepstatin-A had no inhibitory effect. The observed proteinase inhibition by pepstatin-A was thus a genuine effect of the inhibitor rather than a non-specific effect caused by the solvent in which the inhibitor was dissolved.

Table 4.3. The effect of inhibitors on proteinase activity from Pentland Dell tuber tissue. Tubers stored for 18 weeks at 5°C.

Inhibitor	Concentration	Specific activity RFU mg^{-1}(protein) min^{-1} $\bar{x}$ (n=8) ± S.D.	Percentage inhibition
PMSF	Control	5.83 ± 0.20	0
	0.2mM	4.33 ± 0.08	26
	2mM	5.00 ± 1.16	14
	20mM	5.25 ± 0.08	10
Pepstatin-A	Control	4.60 ± 0.23	0
	1μM	0.00 ± 0.00	100
	10μM	0.16 ± 0.16	97
	100μM	0.00 ± 0.00	100
1,10-phenanthroline	Control	6.52 ± 0.17	0
	0.2mM	4.34 ± 0.11	33
	2mM	4.96 ± 0.11	24
	20mM	3.77 ± 0.25	45
E64	Control	4.43 ± 0.54	0
	1μM	4.41 ± 0.20	0
	10μM	5.03 ± 0.82	0
	100μM	4.92 ± 0.20	0
EDTA	Control	11.77 ± 0.23	0
	1mM	6.37 ± 0.23	46
	10mM	9.04 ± 0.00	23
	100mM	13.68 ± 0.68	0
Cysteine	Control	11.77 ± 0.23	0
	1mM	7.01 ± 0.54	43
	10mM	10.62 ± 2.29	10
	100mM	25.34 ± 1.24	0



Inhibition expressed for an enzyme concentration of 1 mg(protein) ml^{-1}.

Figure 4.01. The percentage inhibition of proteinase activity from Pentland Dell tuber tissue as a function of pepstatin-A concentration. Tubers stored at 5°C for 18 weeks.

4.3.2. Purification of tuber aspartic proteinase activity.

Separation of ammonium sulphate-precipitated potato tuber protein by anion exchange chromatography, produced a high level of proteinase activity corresponding with more than one protein peak, this suggesting the possible presence of more than one enzyme (Fig. 4.02.). The active fractions were then pooled (Table 4.4.) and run on a pepstatin A-agarose affinity column. Here, the majority of the protein was not bound to the column and eluted immediately. However, when washed with glycine-HCl buffer pH 2.2, a single peak of bound protein was eluted from the column, this peak containing a high proportion of the proteinase activity (Fig. 4.03. and Table 4.4.). The active fractions were separated on a Sephacryl S-300HR gel filtration column and 3 peaks were eluted (Fig. 4.04.). The first eluted peak (Kav =

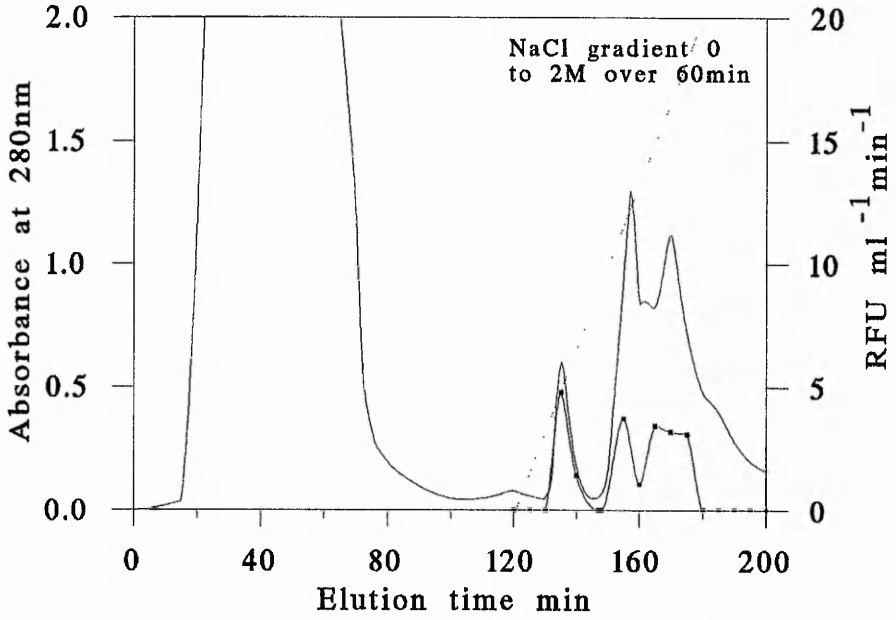


Figure 4.02. Separation of Pentland Dell protein fractions by fast flow Q-Sepharose anion exchange chromatography at a flow rate of 2ml min^{-1}(2ml fractions collected), absorbance at 280nm (—) compared with proteinase activity (▪).

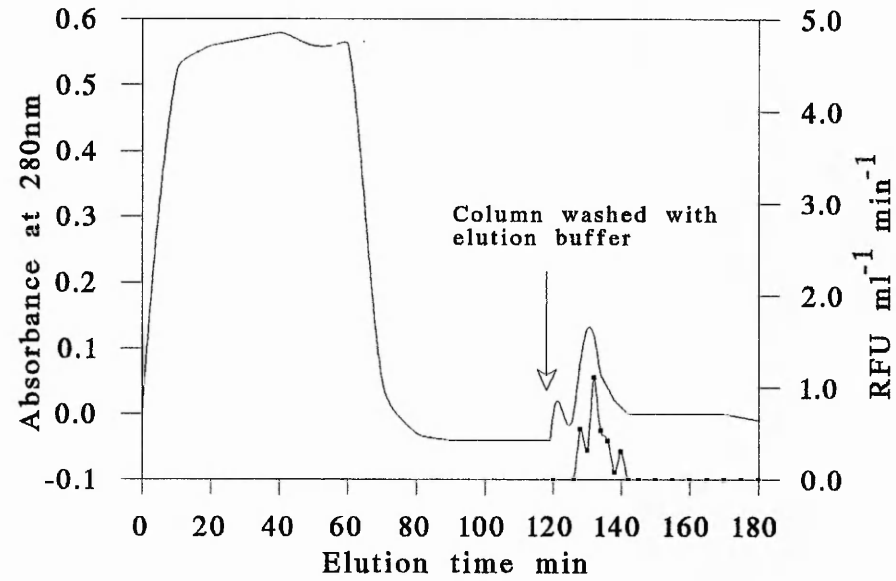


Figure 4.03. Purification of aspartic proteinase activity by pepstatin A-agarose affinity chromatography. Absorbance at 280nm (—) compared with proteinase activity (▪), flow rate 1ml min^{-1} (1ml fractions collected).

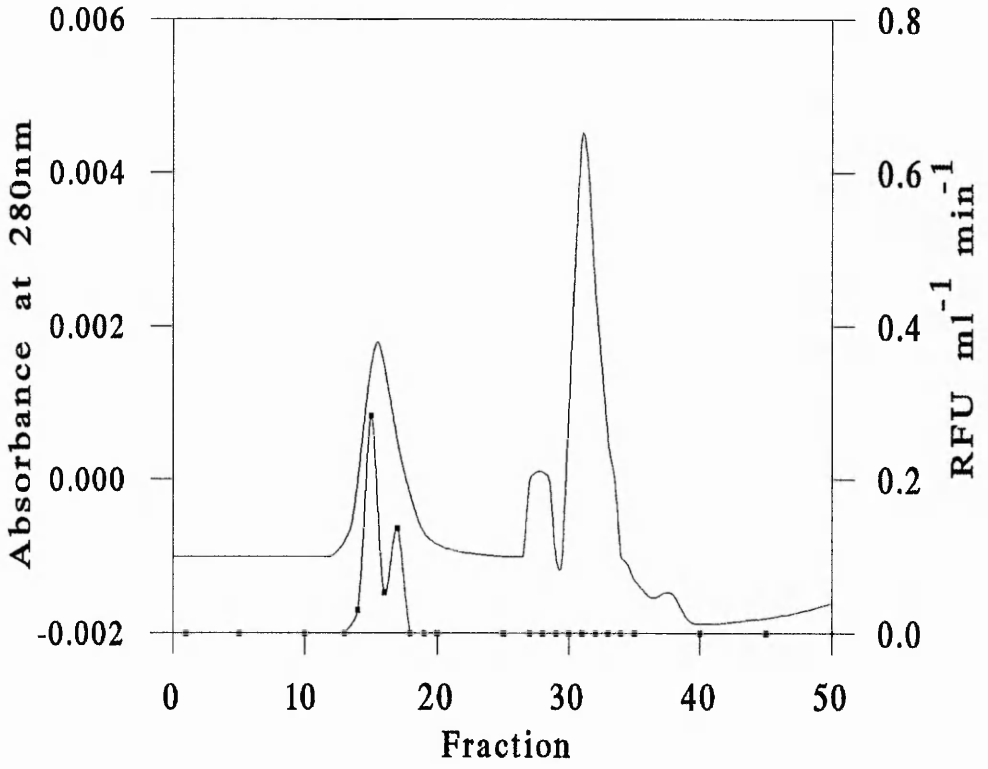
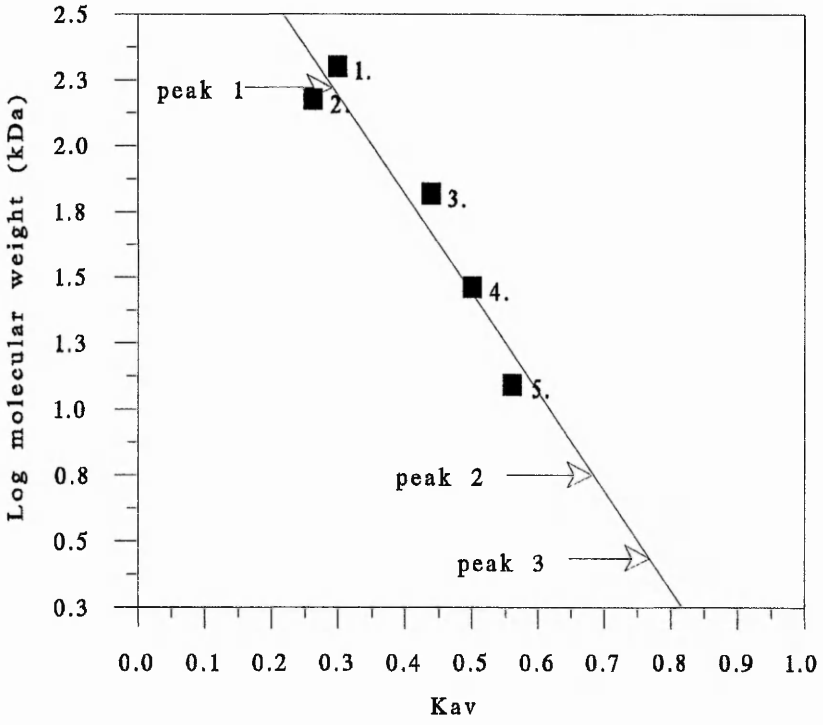


Figure 4.04. Purification of aspartic proteinase activity by Sephacryl S300HR gel-filtration chromatography. Absorbance at 280nm (—) compared with proteinase activity (▪), flow rate 1ml min^{-1} (5ml fractions collected).



1. β-amylase 200kDa
2. alcohol dehydrogenase 150kDa
3. bovine serum albumin 66kDa
4. carbonic anhydrase 29kDa
5. cytochrome C 12.4kDa

Figure 4.05. A calibration curve for the molecular weight of proteins separated using a Sephacryl S-300HR gel-filtration column (column bed volume 250ml)

9.57 x10^{-3}) corresponded with 2 peaks of proteinase activity (Table 4.4.) and had an estimated native molecular weight of approximately 157kDa (Fig. 4.05.). The presence of 2 activity peaks indicated the possible presence of 2 aspartic proteinases. The second absorbance peak (Kav = 0.014) had an estimated native molecular weight of 5.7kDa and the third peak (Kav = 0.015) a weight of 2.7kDa, but neither peak exhibited proteinase activity (Fig. 4.04.). The purification and yield of aspartic proteinase activity is summarised in Table 4.4.

Analysis of the purified aspartic proteinase fraction by SDS-PAGE (Figs. 4.06. and 4.07.) showed a single polypeptide band (Rf = 0.450) displaying an approximate molecular weight of 47kDa.

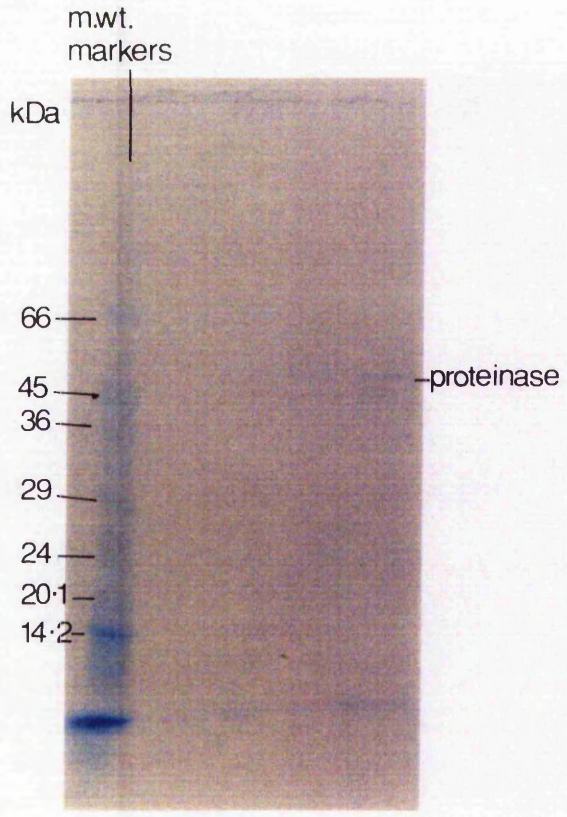
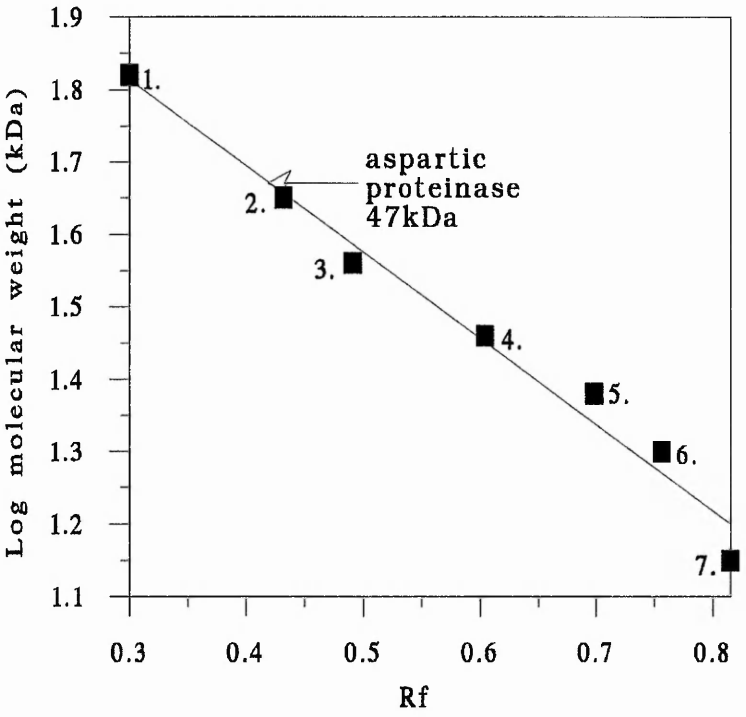


Figure 4.06. Aspartic proteinase molecular weight determination by SDS-PAGE.

Table 4.4. Purification of potato tuber aspartic proteinase.

Purification step	Volume (ml)	Protein (mg ml^{-1})	Total protein (mg)	Total activity RFU min^{-1}	Specific activity RFU mg^{-1}(protein) min^{-1}	Purification factor	Recovery (%)
1. Extraction	720	not assayed		not assayed	not assayed		
2. $(NH_4)_2SO_4$ precipitation	80	1.19	95.20	990.08	10.40	1	100
3. Fast flow Q-Sepharose anion-exchange chromatography	48	1.11	53.28	461.94	8.67	1	47
4. Pepstatin A-agarose affinity chromatography	14	0.09	1.26	36.36	28.86	3	4
5. Sephacryl S300HR gel-filtration chromatography	15	0.01	0.12	61.16	522.8	50	6



1. albumin, bovine 66kDa
2. albumin, egg 45kDa
3. glyceraldehyde-3-phosphate dehydrogenase 36kDa
4. carbonic anhydrase 29kDa
5. trypsinogen 24kDa
6. soyabean trypsin inhibitor 20.1kDa
7. α-lactalbumin 14.2kDa

Figure 4.07. Calibration curve for monomeric molecular weight determination by SDS-PAGE.

To summarise, the tuber proteinase enzyme activity observed throughout the 1992-93 storage season was partially inhibited by the serine protease inhibitor PMSF and the metal chelators, 1,10-phenanthroline and EDTA (Table 4.3.). The cysteine protease inhibitor E64 was completely ineffective. The aspartic protease inhibitor pepstatin-A caused total inhibition of the observed activity at concentrations between 1μM and 100μM (Table 4.3.). A more detailed dose response study showed 50% inhibition (IC_{50}) by 0.15μM inhibitor (Fig. 4.01.). Molecular weight determinations by gel-filtration chromatography and SDS-PAGE revealed the enzyme had approximate native and monomeric molecular weights of 157kDa and 47kDa respectively (Fig. 4.05.). The presence of more than one aspartic proteinase cannot be confirmed from these results.

4.4. DISCUSSION

The 25% inhibition of proteinase activity by PMSF suggested some possible serine protease activity to be present in stored potato tubers (Table 4.3.), an observation consistent with the serine type activity observed by Santarius and Belitz (1978). The serine protease discovered in the latter study exhibited total inhibition by the serine protease inhibitors, DFP (di-isopropylfluorophosphate) and TLCK (tosyllysine-chloromethyl ketone), but was unaffected by TPCK (tosylamide-phenylethyl-chloromethyl ketone) or endogenous trypsin and chymotrypsin inhibitors from potatoes. The proteinase activity observed in the current study was also unaffected by several endogenous trypsin and chymotrypsin inhibitors of the potato tuber, the 22kDa family of inhibitors probably acting as important substrates for this activity (Table 3.1.). This lack of inhibition by endogenous tuber proteinase inhibitors was supported by the results of Kaiser and Belitz (1973), their work suggesting that the tuber proteinase inhibitors are specific for the serine proteases of animals and microbes.

Serine protease activity has been isolated from a variety of plant sources including white gourd (*Benincasa cerifera*) and melon (*Cucumis melo* L.) (Kaneda and Tominaga, 1977; Uchikoba, Niidome, Sata and Kaneda, 1993). The activity was determined in these studies by following the breakdown of protein substrate at 280nm (Kunitz, 1947). Due to the low sensitivity of this type of assay it is likely that the serine protease activity observed was of the proteinase type. However, a large number of studies (Wells , 1965; Zuber and Matile, 1968; Ihle and Dure, 1972; Mikola, 1976; Nishimura and Beevers, 1978) including that of Santarius and Belitz (1978) on potato tubers, have indicated the carboxypeptidase type activity of many plant serine proteases, these peptidases being termed acid carboxypeptidases. The probable serine protease activity observed in this study cannot be confirmed as carboxypeptidase activity due to the use of casein as a substrate.

The complete inhibition of proteinase activity by pepstatin-A (Table 4.3.) indicated that the major proportion of tuber proteinase activity was of the aspartic protease class, the perceived activity also demonstrating an acidic pH optimum typical of the aspartic catalytic mechanism (Fig. 3.27.). This is the first identification of a proteinase from this catalytic class in stored potato tubers. Aspartic or acid proteinases have been identified from seeds of several plant species including soyabean (*Glycine max*) (Bond and Bowles, 1983) and hempseed (*Cannabis sativa*) (St Angelo *et al*, 1969; St Angelo *et al*, 1970), this type of proteinase often being associated with protein bodies. The acid pH optimum of the observed aspartic proteinase activity (Fig. 3.27.) coupled with marked specificity for patatin and the 22kDa protein family (Table 3.1.) both stored in the vacuole (Sonnewald *et al*, 1989; 1990; Suh *et al*, 1990), points to a possible vacuolar location for this proteinase as demonstrated in a variety of plant cells (Matile, 1978; Boller and Kende, 1979; Boller, 1982). If this is the case, it would infer a lysosomal model for storage protein degradation as that in seed protein bodies with the proteinase activity of this aspartic protease initiating the fragmentation of the storage proteins (Ryan, 1973; Wilson, 1986). Subsequent additional cleavage of the polypeptide fragments would possibly occur as a result of carboxypeptidase activity (Wilson, 1986) such as that noted in potato tubers by Hojima *et al* (1971) and Santarius and Belitz (1978). Aminopeptidase activity of tuber tissue (Santarius and Belitz, 1978; Davies and Ross, 1987) may also play a role in this model for storage protein degradation, although because of it's alkaline pH optimum it is likely to have a cytosolic location (Wilson, 1986). The partial inhibitory effect of 1,10-phenanthroline on proteinase activity (Table 4.3.) suggested that the aspartic proteinase is possibly either activated or stabilised by a metal ion. This was further supported by the effect of EDTA with the metal chelator displaying an inhibitory effect at 1mM and 10mM (Table 4.3.).

The lack of proteinase inhibition by the cysteine protease inhibitor, E64 inferred an absence of proteinases with this catalytic mechanism. As cysteine proteinases are

responsible for initial storage protein breakdown in seeds of a variety of plants (Vavreinova and Turkova, 1975; Baumgartner and Chrispeels, 1977; Bulmaga and Shutov, 1977; Shutov and Vaintraub, 1979; Wilson, 1986; Yu and Greenwood, 1994), it is likely that their absence means the prime role in initiating protein breakdown is performed by aspartic proteinase activity, as implied by the high activity against major storage proteins (Table 3.1.). Cysteine demonstrated inconsistent effects on activity (Table 4.3.), further advocating a lack of cysteine proteinase activity in tuber tissue. The partial inhibition by 1mM and 10mM cysteine suggested the proteinase activity is sensitive to reducing agents, although this was contradicted by the 215% activation with 100mM cysteine.

The SDS-PAGE separation of the purified tuber aspartic proteinase fraction was unable to confirm the presence of more than one proteinase with this catalytic mechanism, as only a single polypeptide band was evident (Figure 4.06.). The monomeric molecular weight of approximately 47kDa calculated from SDS-PAGE was consistent with those reported for other enzymes of this catalytic class, such as fungal aspartic proteases, which generally exhibit molecular weights in the range of 30kDa to 45kDa and consisting of approximately 327 amino acid residues (Barrett, 1986; Creighton, 1993). These studies have demonstrated that aspartic proteases are predominantly monomers made up of two identical domains (Creighton, 1993), indicating that the native molecular weight of 157kDa determined by gel-filtration may be as a result of aggregation and thus cannot be confirmed.

4.5. CONCLUSIONS

The following conclusions may be drawn from the results detailed in this Chapter.

Firstly, the primary proteinase responsible for degrading tuber storage proteins such as patatin and the 22kDa protein family is of the aspartic catalytic class. This enzyme, possibly stabilised or activated by a metal ion, has a probable monomeric structure with a molecular weight of 47kDa.

Secondly, the class of activity, pH optimum and substrate specificity suggest that this aspartic proteinase is a vacuolar enzyme responsible for initiating reserve breakdown. Later stages of amino acid liberation are probably performed in the vacuole by acid carboxypeptidases and in the cytosol by aminopeptidases.

Finally, a low level of serine protease activity was identified in tuber tissue, the literature implying this may be of the acid carboxypeptidase type.

CHAPTER 5. DISCUSSION.

5.1. ASSESSMENT OF THE METHODOLOGY.

Any data is only as good as the supporting methodology and this thesis is no exception. As a consequence, an honest appraisal of the experimental approaches and methods used in this study has been performed. This assessment of the methodology is detailed below.

Although metabolite analyses and enzymes assays were performed on tubers of the same population to those used for fry colour determination, identical tubers were not used for both studies at each sample time. The use of separate tuber samples for metabolite/ enzyme activity determinations and fry colour measurement was necessary as fry colours were determined at Sutton Bridge Experimental Station whereas all other studies were carried out at The Nottingham Trent University. As a result, the degree of variation within the population may have resulted in some discrepancies when comparing reducing sugar/ amino acid contents and the respective fry colours. In order to minimise such differences, mean values for metabolite concentrations and fry colours were used for all comparisons.

Basal tuber-core sections were utilised for the extraction of free amino acids, soluble proteins, reducing sugars and enzyme activities, whereas whole tubers were used for fry colour determinations. It is possible that the metabolite concentrations of the basal core section do not totally reflect those of the entire tuber. This may have resulted in further discrepancies in the comparisons of metabolite concentrations with fry colour. In addition, the metabolic trends of these basal core sections may not represent the overall metabolic activity of the tuber during storage. Earlier studies (Hart *et al*, 1986; Hart and Cobb, 1988) suggested that changes in storage of metabolite contents were most marked in basal and median core sections, whilst relatively little change of

metabolism occurred in the apical section. It was hence decided to focus on the basal core section as degradation of storage reserves in this portion of the tuber would undoubtedly affect the fry colour of the entire tuber. It was also envisaged that using the portion of the tuber most affected by the storage conditions would enable a clearer picture of storage metabolism to be developed.

The sampling times for the above biochemical studies and fry colour determinations did not always coincide throughout each storage season. As a result, on several occasions no fry colour values were obtained for comparison with the measured reducing sugar and amino acid concentrations. These points during each storage season were thus not included in correlations of reducing sugars/ amino acids with fry colour.

At each sampling point performed for the measurement of metabolite concentrations and enzyme activities, the total free amino acid and individual amino acid contents were determined by ninhydrin assay and HPLC techniques respectively. Because of the different nature of these assay techniques, some variation in the relative accuracies of these methods may have been experienced. The ninhydrin assay for estimating total free amino acid content was calibrated by the use of a range of concentrations of the amino acid, glycine. As this single amino acid was used to calibrate for a range of amino acids comprising the total pool, it is likely that a degree of inaccuracy was inherent with this technique. Quantification of individual amino acids by reverse-phase HPLC using OPA-2-mercaptothanol derivatisation, would have enabled a more accurate estimate of total amino acid to be determined from the sum of the individual contents. However, this method was deemed too time consuming for the routine measurement of total free amino acids. In addition, assays of amino acid content by techniques based on both ninhydrin and OPA-2-mercaptoethanol were unable to detect secondary amino acids such as proline and hydroxyproline. This drawback was considered of relatively minor importance as the literature indicated that secondary

amino acids comprise only a small proportion of the total amino acid pool (Davies, 1977; Synge, 1977).

The soluble protein contents of tuber tissue and enzyme extracts were measured by the Coomassie Blue dye-binding method of Bradford (1976) throughout each storage season. The technique involved the use of bovine serum albumin (Sigma, Fraction V) as the calibration standard. As this non-tuber protein may display a variety of differences to those present in the tuber protein pool, the method can only provide an estimate of protein concentration due to variation in colour formation between proteins. However, other non-specific protein determination procedures such as the Bicinchoninic Acid assay and measurement of absorbance at 280nm also suffer from protein to protein variability in their response (Smith *et al*, 1985). The Bradford method is also simple, relatively sensitive and unaffected by buffer or enzyme assay components except detergents such as SDS (Bradford, 1976). This technique may have been improved through the use of an endogenous tuber protein such as patatin as the calibration standard. However, the high degree of purity required for such a protein standard would have required a time consuming extraction and purification procedure, this being deemed impractical within the time-scale of this study.

A drawback of the metabolite extraction and protein assay techniques used in this investigation, is that they do not allow the measurement of changes in the insoluble protein content of tubers. However, this may be determined by the Kjeldahl total nitrogen assay to provide a complete picture of protein metabolism during storage. Previous studies have showed the low insoluble protein content to be concentrated in the outer layers of the tuber, these varying only slightly during storage (Neuberger and Sanger, 1942; Racusen, 1983).

The proteinase activity in tuber tissue was measured throughout the 1992-93 storage season using the FITC-labelled non-tuber protein casein as the substrate. However, it

is possible that the observed activity may not have entirely reflected that occurring in tuber tissue due to the pronounced substrate specificity for endogenous tuber proteins. A more accurate overview of proteolysis during storage would have undoubtably been provided using a tuber protein such as patatin as the substrate. Although, due to the time consuming nature of protein purification and the large quantities required, the use of a commercially available protein such as casein as the substrate was the most practical alternative. As the same batch of FITC-casein was used throughout storage as the substrate, it is hoped that any variation in activity due to the nature of the substrate was minimised.

Inhibitor studies performed at 18 weeks in storage showed that proteinase activity was primarily of the aspartic proteinase class, though the types of activity present may have altered during the storage season. However, results from preliminary inhibitor studies and the purification of the aspartic proteinase performed at 13 and 36 weeks respectively, suggested that this form of activity is the predominant proteinase throughout the majority of storage. This would indicate that very little variation in observed activity due to differing substrate specificities would occur during storage.

All tubers used in this study were treated with the sprout suppressant chemical CIPC. The relative lack of sprout formation resulting from this treatment inferred a low sink strength in the possible source-sink relationship between tubers and sprouts (Davies and Ross, 1984). It is hence likely that the mobilisation of nitrogen reserves by proteinase activity never reached it's maximum potential in the tubers used in this study. In addition, the transport of free amino acids to the external tissues of the tuber may have been suppressed by CIPC (Nowak, 1977). In order to provide a clearer picture of the trends associated with reserve breakdown and translocation, it would be neccessary to carry out storage experiments in the absence of CIPC treatment, this allowing unabated sprout formation. However, as sprout inhibitors are universally

applied in the storage of tubers for processing, the relevance of such a study in explaining the decline of processing potential during storage is unclear.

The primary enzymes of amidation (GS) and deamidation in non-green tissue (NADH-GOGAT) were assayed throughout storage in 1992-93. Although NADH-GOGAT is probably the key enzyme of glutamine deamidation in potato tuber tissue as in a variety of seeds and tubers (Beevers and Storey, 1976; Dilworth and Dure, 1978; Salisbury and Ross, 1978; Osuji *et al*, 1991), it is possible that ferredoxin-dependent GOGAT may also play a role. For a complete study of amidation and deamidation it would thus be necessary to study not only glutamine synthetase and NADH-GOGAT, but also Fd-GOGAT. In addition, asparaginase may play a key role in the deamidation of asparagine and as a consequence, this enzyme would also need to be assayed throughout tuber storage.

It is hoped that the majority of criticisms have been satisfactorily overcome, however given the various constraints imposed by the nature of this study, practicalities have been considered when designing experimental approaches.

5.2. INTERPRETATION OF RESULTS IN RELATION TO THE LITERATURE

The metabolic activity of stored tubers may be divided into three phases, these generally being associated with early, mid and late storage although their durations varied from season to season.

Upon entering storage, tubers exhibited a fairly high rate of metabolic activity, this may be at least in part due to the high temperatures required for the curing process. This heightened rate of metabolism at the beginning of storage was confirmed by studies on tuber respiratory rate (Williams and Cobb, 1992; 1993). The metabolically

active nature of the tubers was reflected in both Pentland Dell and Record, by the high rate of proteinase activity observed in the 1992-93 season and NADH-GOGAT activity in the 1991-92 season (Figs. 3.29., 3.30., 3.37. and 3.38.). During this initial stage of storage, the temperature regime had little influence on the rate of respiration and hence nitrogen metabolism *per se*, as a result, the concentrations of free amino acids and soluble proteins were unaffected by storage temperature (Williams and Cobb, 1992; 1993).

In several instances the metabolically active nature of the tubers resulted in a breakdown of soluble protein over this early phase of storage (Figs. 3.03., 3.05. and 3.07.). As this was not accompanied by an upturn of free amino acids, it is likely that these metabolites may be translocated from the tuber core to external tissues (Cotrufo and Levitt, 1958). However, in some seasons total soluble protein was observed to increase over this period, in 1991-92 the rise coinciding with a slight decline of patatin (Figs. 3.06., 3.08., 3.20. and 3.22.). This suggested that protein turnover took place during this initial stage of storage (Fig. 5.01). The net direction of nitrogen flux probably depending on a variety of factors including physiological maturity and the metabolic state of the tuber at the start of storage, these differing between storage seasons and cultivar (Burton, 1969; Sowokinos, 1978).

The high amide proportion of the free amino acid pool over this early period of storage (50-90%) may result from a combination of processes. These include the extent of nitrogen fertiliser application during tuber growth and the balance of nitrogen flux both before and following harvest. The application of increased fertiliser rates has been demonstrated to increase free amino acids and elevate the proportion of the free amino acid pool consisting of asparagine and glutamine, whilst also lowering reducing sugar content (Millard, 1986; Roe *et al*, 1990). A high rate proteolysis coupled with glutamine synthetase activity may result in further amino acid amidation (Fig. 5.01.).

At this initial stage of the storage season, the free amino acid pool appears to have little influence on tuber processing potential in either Pentland Dell or Record (Figs. 2.43. to 2.45. and 2.47. to 2.51.). In all seasons, low-temperature sweetening occurred, possibly as a result of reduced respiration due to the onset of dormancy, invertase activation and the cold lability of phosphofructokinase (Tishel and Mazelis, 1966; Van Es and Hartmans, 1987). This rapid increase in reducing sugar content at 5°C appears to be the sole factor causing the early season decline in processing potential.

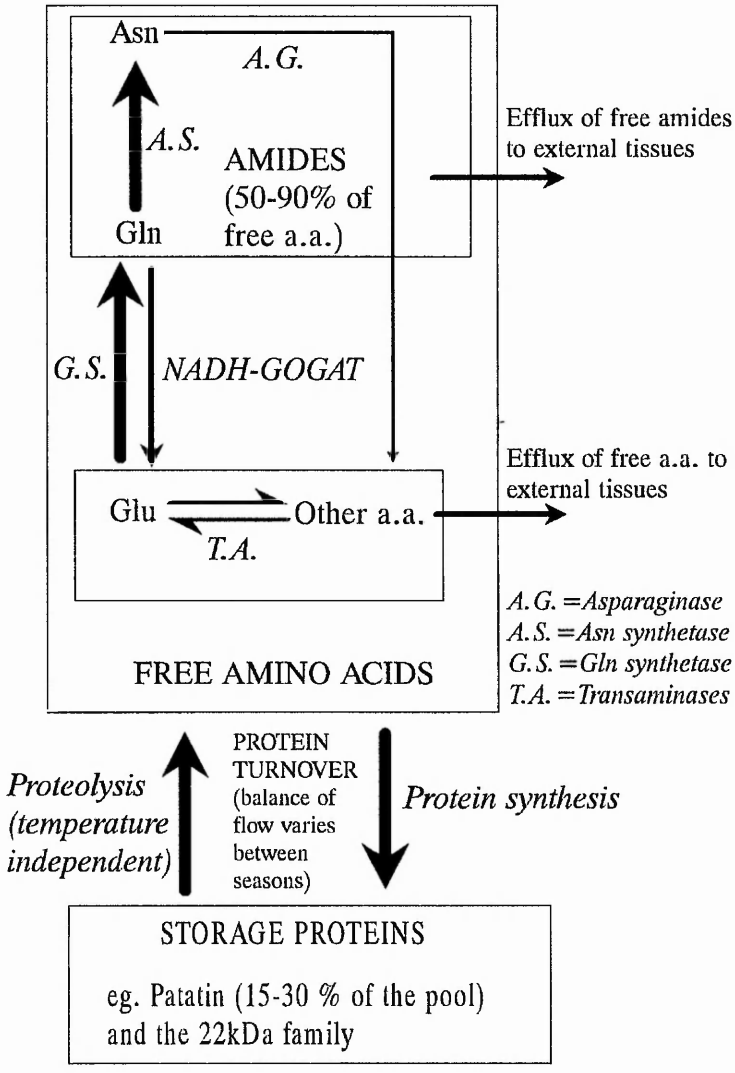


Figure 5.01. A model to demonstrate nitrogen flow in tuber core tissue during the metabolically active initial phase of storage.

On becoming fully dormant, tubers enter the second general phase of storage which lasts until the end of the rest period. This dormancy phase is associated with a low degree of metabolic activity, respiration remaining at a minimal rate irrespective of storage temperature throughout this period (Williams and Cobb, 1992; 1993). As a result, proteinase activity was low throughout dormancy as noted by Nowak and Skwiercz (1975), this trend being independent of storage temperature in the 1992-93 season (Figs. 3.28., 3.29. and 5.2.). During this phase of storage, the balance of nitrogen flow was in the direction of net protein synthesis, particularly of those with low molecular weights (Levitt, 1954; Cotrufo and Levitt, 1958; Ashford and Levitt, 1965; Nowak,1977). In the present study, the free amino acid pool generally remained at a low size at both 5° and 10°C. As a result, it is likely that an inward movement of free amino acids from the external tissues of the tuber was required to supply nitrogen for this synthesis of protein (Cotrufo and Levitt, 1958). The study of protein changes during dormancy by Nowak (1977) showed low temperatures to curtail synthesis, this explaining the mid-season temperature effects observed in Figures. 3.04., 3.05. and 3.07.

Due to the lack of a temperature influence on proteinase activity during this phase of storage, it may be inferred that higher storage temperatures direct the balance of protein turnover towards a greater net synthesis. If this is the case, then short-term reconditioning treatments would be expected to elevate the soluble protein content of tuber core tissue. However, the absence of an obvious effect of reconditioning implies that proteinase activity may also be enhanced by these treatments, possibly due to the breaking of tuber dormancy (Figs. 3.04. and 3.07.). The proportion of the free amino acid pool consisting of the amides tends to undergo a slight drop during tuber dormancy to between 50 and 60% (Figs. 2.20., 2.21., 2.22., 2.28., 2.29. and 2.30). This decline may be a consequence of the influx of free amino acids into the tuber core coupled with deamidation by NADH-GOGAT and asparaginase, these enzymes providing a supply of non-amidated amino acids for protein synthesis. In addition,

protein synthesis may also contribute directly to the decline in the amide fraction by the incorporation of some asparagine and glutamine residues into the newly synthesised proteins (Fig. 5.02.). The main tuber storage proteins such as the 22kDa family and patatin both containing a relatively high proportion of these amides (Gorenstein *et al*, 1988; Suh *et al*, 1990).

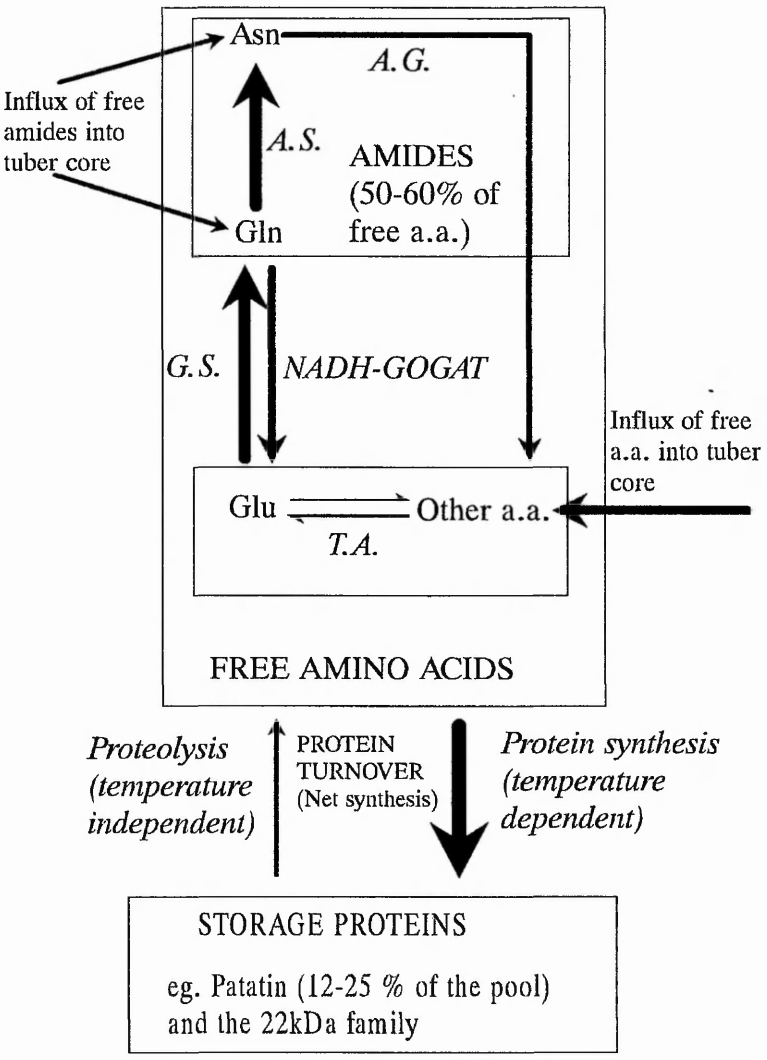


Figure 5.02. A model to demonstrate nitrogen flux in tuber core tissue during the dormant phase of storage.

When processed, tubers in this dormant phase of storage demonstrate little influence of free amino acid content on fry colour production (Figs. 2.43. to 2.45. and 2.47. to

2.51.). Low storage temperatures resulting in poor fry colours, purely as a consequence of low-temperature sweetening at the end of the initial period of storage. It is hence likely that improvement of fry colours through the use of short-term reconditioning treatments, operates solely through the decrease of reducing sugars. The lowering of these Maillard reactants is brought about by either a burst in respiration or reconversion into starch (Heulin and Barker, 1939; Schippers, 1977; Williams and Cobb, 1992; 1993).

The final phase of tuber storage metabolism is associated with a marked rise of metabolic activity brought about by the break of dormancy (Fig. 5.03.). This upturn in metabolism is reflected by increases in the rate of respiration and proteolysis (Nowak and Skwiercz, 1975; Williams and Cobb, 1992; 1993). In the present study, heightened rates of proteinase activity resulted in a rise of free amino acid content due to the mobilisation of nitrogen storage reserves, the trend increasing with temperature in Pentland Dell, though not in Record in 1992-93 (Figs. 3.29., 3.30. and 5.03.). This degradation of storage proteins probably had the function of supporting sprout initiation and growth (Levitt, 1952; Levitt, 1954; Cotrufo and Levitt, 1958; Nowak and Skwiercz, 1975; Nowak, 1977; Davies and Ross, 1984; Davies and Ross, 1987). As a consequence, free amino acids from this mobilisation may be translocated from the tuber core to the eyes (Cotrufo and Levitt, 1958), this possibly occurring in Record tubers in 1990-91 (Figs. 3.07. and 5.03.).

In several cases in the present study, the rise of free amino acids over this final period was not matched by a decline of storage proteins, thus suggesting the occurrence of protein synthesis as a continuation of that taking place in the dormant phase (Figs. 3.02., 3.04., 3.05, 3.06., 3.09. and 5.04.). Such a synthesis in conjunction with storage protein degradation and a rise of amino acids would infer a continued influx of free amino acids into the tuber core (Fig. 5.04.). This translocation of free amino acids in the opposing direction to that required for sprout growth may result from CIPC

treatment, this chemical inhibiting the outward transport of nitrogen to the eyes (Nowak, 1977). The inhibition may be explained in terms of the source-sink relationship between tubers and sprouts, tuber eyes lacking sprout formation having a low sink strength compared to those with developing sprouts (Davies and Ross, 1984).

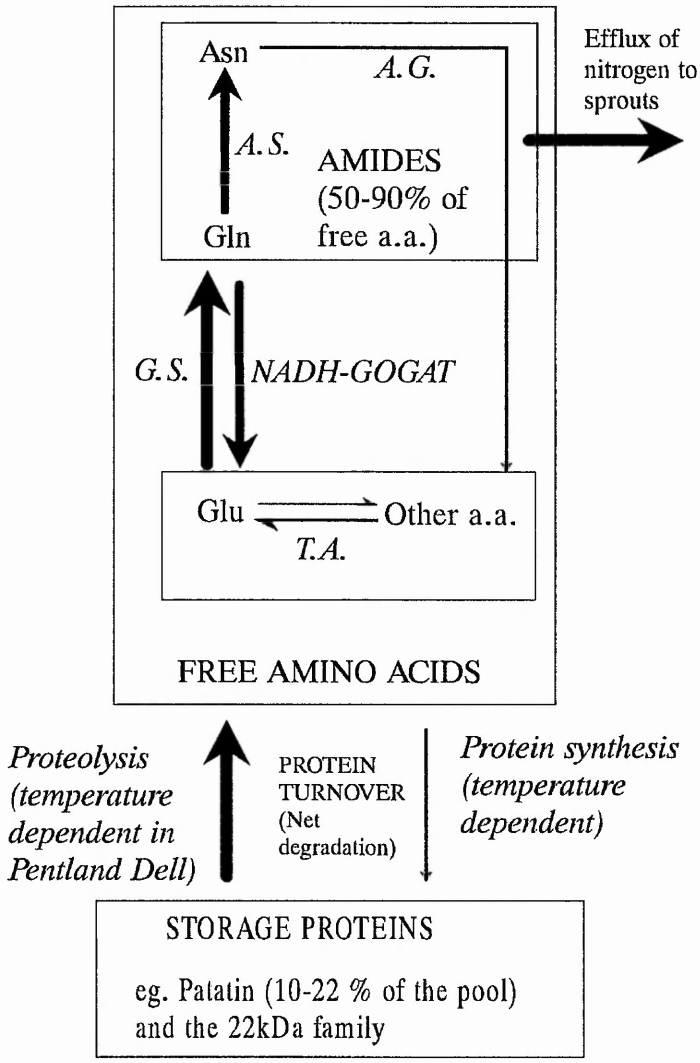


Figure 5.03. A model to demonstrate nitrogen flow in tuber core tissue, subsequent to the break of dormancy in tubers undergoing sprout formation.

The major storage protein groups, patatin and the 22kDa family are both located in the vacuoles of tuber cells (Sonnewald *et al*, 1989; 1990). In addition, the 47kDa aspartic proteinase characterised from tuber tissue also has a probable vacuolar

location as shown for similar enzymes in cells of other plant species (Matile, 1978; Boller and Kende, 1979; Boller, 1982). The tuber cell vacuole may thus perform a similar lysosomal function as the protein bodies of seeds, these containing the bulk of storage protein and a high proportion of proteinase activity (St Angelo *et al*, 1969; Tully and Beevers, 1978; Baumgartner *et al*, 1978). This possible lack of enzyme-substrate separation does not support the control of reserve mobilisation by enzyme compartmentation as suggested for tuber starch metabolism by Bailey *et al* (1981). The increase of proteolysis and subsequently free amino acids on the ending of dormancy may thus result from *de novo* synthesis of the proteinase (Fig. 5.05.). A similar rise in proteinase activity occurs on the germination and seedling development of a variety of plant seeds, an example being the *de novo* synthesis of vicilin peptidohydrolase in mung bean (*Vigna radiata*) seeds (Chrispeels *et al*, 1976).

The growth of the reducing sugar pool in this late phase at higher storage temperatures is a result of starch hydrolysis. Senescent sweetening is possibly initiated by the increasing permeability of the amyloplast membrane, this removing the intracellular compartmentation of starch from hydrolytic enzymes (Workman *et al*, 1979). The rise in reducing sugar content during this phase of storage hence requires membrane leakiness, in contrast to the increase of free amino acids (Fig. 5.05.). As a result these processes may not always coincide, the rise in free amino acids in Pentland Dell stored at 10°C occurring before that of reducing sugars (Figs. 2.44. and 2.45.). In these situations, the fry colour of processed tubers deteriorates in association with growth of the free amino acid pool. Senescent sweetening does not take place until after this reduction in processing potential and in 1992-93, no rise in reducing sugars was observed in 10°C Pentland Dell (Fig. 2.47.). The downturn of processing potential caused by the increase of free amino acids was demonstrated by the occurrence of a heightened fry colour production per unit reducing sugar as noted for high amino acid content tubers by Hughes and Fuller (1984). This probable synergistic influence of free amino acids on fry colour as proposed by Roe *et al* (1990) only

occurred in Pentland Dell at 10°C, due to the greater upturn of proteinase activity at this temperature (Figs. 2.47. and 3.29.).

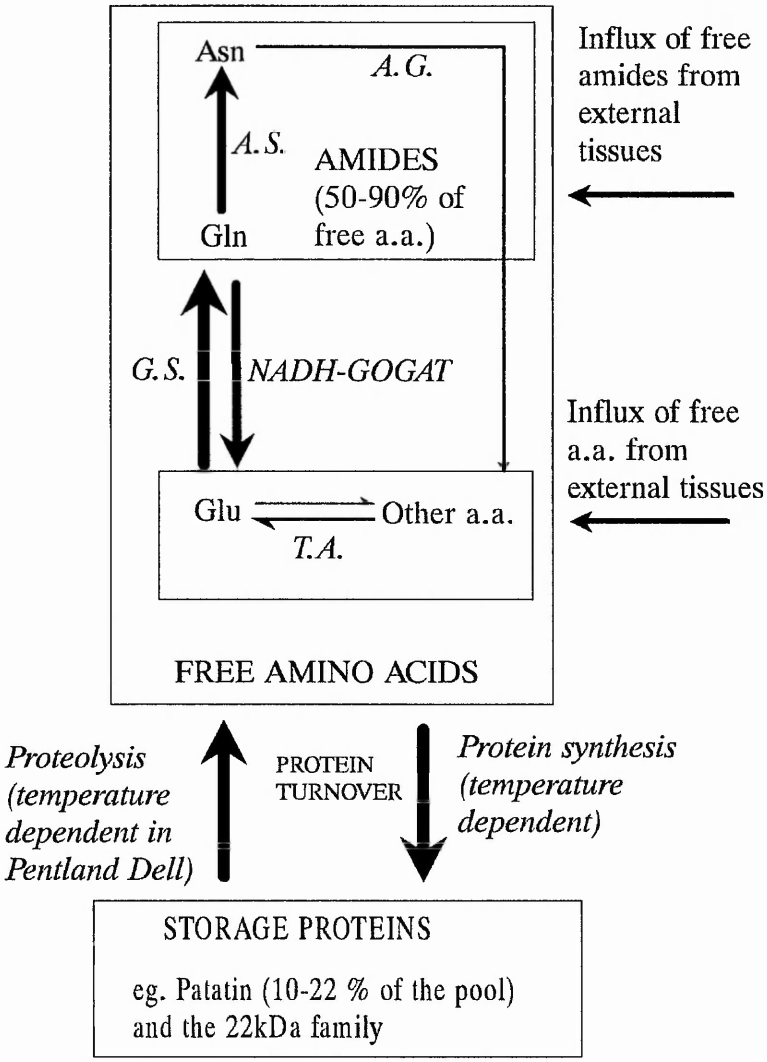


Figure 5.04. A model to demonstrate nitrogen flow in tuber core tissue, subsequent to the break dormancy in tubers with restricted sprout formation.

Despite a relatively large late-season increase of free amino acid content at 5°C in both Pentland Dell and Record, these metabolites did not appear to influence fry colour production at this temperature. This infers that the poor processing potential of low-temperature stored tubers was due soley to their elevated reducing sugar content. It is possible that an increase of free amino acids at 5°C was unable to darken fry

colours further than the degree already achieved, by high reducing sugars in the absence of this rise in amino acids.

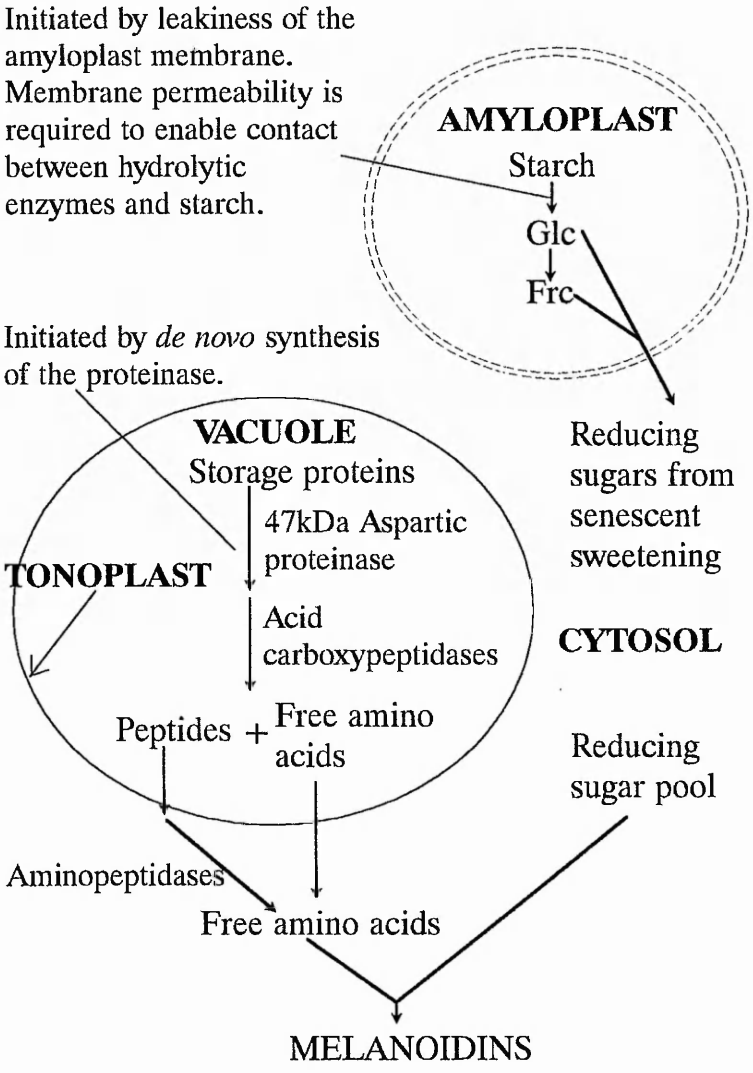


Figure 5.05. Metabolic factors resulting in the late-season deterioration of processing potential in high temperature stored tubers (eg. Pentland Dell stored at 10°C).

The fry colour of Record at 10°C was not observed to deteriorate markedly over the the final phase of storage in any season of this investigation. As a consequence, the effect of the late-storage amino acid increase on processing potential remains unclear in these tubers. The lack of a clear influence of amino acids on fry colour in Record at 10°C points to another as yet undetected factor that may regulate amino acid

importance. This factor may differ between Pentland Dell and Record, hence resulting in the disparity of amino acid influence on processing potential observed between these cultivars.

The use of reconditioning to improve fry colour is often ineffective during this late period of storage due to the irreversible nature of senescent sweetening (Workman *et al*, 1979; Van Es and Hartmans, 1987). In addition, reconditioning is also ineffective as a means of altering the net direction of nitrogen flux, the upturn of free amino acids after the break of dormancy occurring regardless of storage temperature. However, increases of storage temperature may affect the rate of proteinase activity as noted for Pentland Dell in 1992-93 (Fig. 3.29.). As a result, late-season reconditioning treatments may have some adverse effects on the determinants of fry colour through the elevation of the free amino acid content.

The majority of the free amino acid upturn associated with the break of dormancy may be attributed to asparagine, glutamine and to a lesser extent aspartate and glutamate (Figs. 2.18., 2.22., 2.39. and 2.41.). The amides accumulate primarily due to the action of glutamine synthetase and subsequently asparagine synthetase, although a fraction of the increase was probably caused by the direct release of amide residues from storage proteins (Gorenstein *et al*, 1988; Suh *et al*, 1990). It is possible that the rate of proteinase activity is the factor limiting amide formation as this controls the availability of free amino acids for amidation (Figs. 3.31., 3.32., 5.03. and 5.04.). The relative abundance of both the amides was probably a consequence of their role as nitrogen transport compounds for the movement of nitrogen reserves to the developing sprouts. This process demonstrating a degree of similarity to that in a variety of germinating seeds (Streeter, 1973; Capdevilla and Dure, 1977; Dilworth and Dure, 1978). Due to their relative abundance, the amides are significant factors in the determination of fry colour intensity in 10°C Pentland Dell. However, they do not influence processing potential to a greater extent than the total amino acid content

(Table 2.8.), this being consistent with the findings of Roe *et al*, 1990. As amide metabolism is rigidly controlled during storage, neither asparagine nor glutamine can be attributed a greater significance. This contradicts the key role of either asparagine or glutamine proposed in earlier studies (Hart and Cobb, 1988; Cobb *et al*, 1990; Khanbari and Thompson, 1993).

5.3. FUTURE EXPERIMENTS

The following experiments are proposed for the continuation of this study.

Firstly, there is a need to continue the examination of the soluble protein pool during storage. Investigation of the composition of this pool throughout the season by SDS-PAGE and FPLC would enable the individual protein fractions responsible for key changes in the free amino acid pool to be determined. In conjunction with the assay of proteinase activity, this would enable a precise picture of the association between proteolysis and the composition of the protein pool to be developed. The extent of protein synthesis in tuber tissue during storage may also be investigated through the use of ^{35}S-methionine. This would be applied to either whole or part tubers in an incubating solution, at intervals throughout storage. Such an investigation would provide information about the protein species synthesised when used in conjunction with protein separation techniques such as FPLC and SDS-PAGE. A more non-invasive approach would be to investigate the overall fate of nitrogen during tuber growth, harvesting, storage (5° and 10°C) and reconditioning, through the fertilisation of potato plants with ammonium nitrate containing ^{15}N. In order to distinguish between individual labelled proteins and other nitrogen containing compounds FPLC, SDS-PAGE and protein assay techniques would be applied. When combined with studies on the rate of fertiliser, and CIPC application and ^{15}N incorporation into melanoidins upon processing, this would enable a more precise model of nitrogen flux

and its effect on fry colour to be developed. Labelled melanoidins may be separated from tuber metabolites by HPLC and identified by NMR and mass spectroscopy.

In addition, further studies may be performed on the aspartic proteinase activity of potato tubers. Both the kinetic and structural properties of the isolated 47kDa proteinase would be examined, the latter including the primary structure by protein sequencing and the secondary and tertiary structures by X-ray crystallography. This characterisation would aid comparison of the 47kDa proteinase with other enzymes of the aspartic proteinase class. When correlated with structural data on potato storage proteins, the primary and secondary structures of the proteinase would also enable the development of a greater understanding of the enzyme's substrate specificity.

The isolated 47kDa proteinase may also be used to raise a monoclonal antibody, this having several applications. Through the attachment of a fluorescein marker to the antibody, the intracellular location of the proteinase may be confirmed by the use of immunofluorescent microscopy techniques. Alternatively, the localisation and relative abundance of the proteinase may be examined using immuno-gold electron microscopy techniques. This study would enable an accurate relationship between the proteinase abundance and activity to be developed, so establishing the relative importance of *de novo* proteinase synthesis in the mobilisation of nitrogen reserves.

The monoclonal antibody may also be used to quantify the proteinase content through ELISA. This has a practical application in enabling the prediction of a decline in processing potential caused by accumulation of free amino acids. The use of an ELISA based kit to determine *de novo* proteinase increases in a store situation would enable processors to remove tubers from storage before a significant rise of amino acids could take place. The crop could hence be processed immediately whilst still of an acceptable quality, this avoiding the expense and wasteage caused by discarding a

high proportion of the product upon processing. Future experiments could involve the development of such a kit and evaluation in an experimental store situation.

5.4. CONCLUSIONS OF THE INVESTIGATION

The following conclusions may be drawn from the results of this investigation.

The free amino acid content of stored tubers is controlled by a combination of proteinase activity and protein synthesis. The balance of these factors varies according to the metabolic stage of tuber storage, the overall pattern of metabolism showing similarity in all cultivars. In general, tubers demonstrate a high metabolic activity on entry into storage, the direction of nitrogen flow differing between storage seasons. During tuber dormancy the direction of nitrogen flux is shifted towards protein synthesis in the tuber core, this maintaining the resting-state by rendering free amino acids unavailable for sprout initiation. Finally, the break of dormancy is associated with an upturn in the activity of a 47kDa aspartic proteinase in the vacuole, probably due to *de novo* synthesis of the enzyme. Consequentially, the end of the dormancy period results in a switch of nitrogen flow towards the accumulation of free amino acids to support sprout formation. A variety of storage protein species are present in the vacuole of tuber cells, no single protein demonstrating the primary role as the source of free amino acids. The predominant amino acids responsible for this upturn are the amides, asparagine and glutamine due to their role as transport compounds. As amide residues from storage proteins do not account for the entire rise, the activities of glutamine synthetase and asparagine synthetase are instrumental in the accumulation of these metabolites.

The temperature of tuber storage does not affect the overall balance of nitrogen flow at any stage in storage. Short-term reconditioning treatments are hence ineffective as a means of reducing the free amino acid pool size, these treatments operating solely through the lowering of reducing sugar content. When the flux of nitrogen exhibits a distinct direction this may be further enhanced by increased storage temperatures, providing there is no change in the metabolic state of the tuber. As a result, the

magnitude of the free amino acid accumulation on the break of dormancy in Pentland Dell is dependent on storage temperature.

The low reducing sugar content of high temperature stored tubers coupled with the end of dormancy accumulation of free amino acids, results in a synergistic influence of amino acids on fry colour production in Pentland Dell. In this instance, fry colour deteriorates in association with the increase of free amino nitrogen, no upturn of reducing sugar content being required for this reduction in processing potential. The amides are of prime importance as fry colour determinants in Pentland Dell due to their abundance in the latter stages of storage, although no individual amide may be attributed a key role. The results of this investigation indicate that low storage temperatures are advantageous in Pentland Dell by maintaining a low free amino acid content during late storage. However, the benefits of such a regime are more than outweighed by the negative effects of low-temperature sweetening. In low temperature stored Pentland Dell and in Record at all temperatures, reducing sugars alone provide the most accurate measure of processing potential. This is due to the presence of these Maillard reactants at a limiting concentration in tuber tissue. As a consequence, optimal Maillard reactant concentrations for processing may be maintained by high storage temperatures up until the end of dormancy. Beyond this point in storage the extent of proteolysis may be crucial factor in determining processing potential. This infers the need for an ELISA kit to predict the onset of amino acid accumulation and allow the removal of tubers from storage while still of a suitable quality for processing.

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APPENDIX

Calculation of individual free amino acid contents.
The concentrations of individual amino acids in tuber tissue were calculated as follows.

Concentration from HPLC (μM) x Dilution factor of 100 = Amino acid concentration from dissolving 4mg freeze-dried extract in 10ml pure water

x 2.5 = μmoles(amino acid) g^{-1}(freeze-dried extract) l^{-1}

x 10^{-6} x molecular wt. of amino acid x 1000 = g(amino acid) g^{-1}(freeze-dried extract)

x (freeze-dried extract wt./ fresh wt. of tuber tissue used in extraction procedure)

= g(amino acid) g^{-1}(fresh wt.)

g(amino acid) g^{-1}(fresh wt.) x 1000 = mg(amino acid) g^{-1}(fresh wt.)

Calculation of reducing sugar contents.
Individual reducing sugar contents of tuber tissue were calculated by the following procedure.

The absorbance change at 340nm over each assay step corresponded to the concentration of a particular sugar (1. glucose, 2. fructose, 3. sucrose). This change was converted directly to the appropriate sugar concentration (μg in each microplate well) using a regression coefficient determined from the assay of sugar standards.

Sugar concentration expressed as μg(sugar) microplate $well^{-1}$ x Dilution factor of 150

= μg(sugar) g^{-1}(freeze-dried extract)

x (freeze-dried extract wt./ fresh wt. tissue used in extraction procedure)

= μg(sugar) g^{-1}(fresh wt.)

μg(sugar) g^{-1}(fresh wt.)/ 100 = mg(sugar) g^{-1}(fresh wt.)

Assay of lipid acyl hydrolase activity.

LAH activity was assayed by the method of Turnbull and Cobb (1992). Freeze-dried tuber core samples were ground with 15ml 100mM potassium phosphate buffer pH8.0 and subsequently centrifuged at 45000g for 20min. The LAH activity of the supernatant was determined using p-nitrophenyl palmitate as substrate, this lipid being

solubilised with Triton X-100. The assay mixture contained 500µl of 2mM p-nitrophenyl palmitate, 100µl of crude enzyme extract and 1.4ml 500mM potassium phosphate buffer pH 8.0, all incubated at 25°C. The reaction was monitored spectrophotometrically at 400nm, activity being expressed as µmoles p-nitrophenol mg^{-1}(protein) min^{-1}.

Assay of trypsin inhibition by potato protein fractions.

The trypsin inhibitory properties of potato proteins were determined using a modified version of the method of Erlanger, Kokowski and Cohen (1961). The reaction mixture consisted of 100µl 1mM Nα-benzoyl-DL-arginine-paranitroanilide (Sigma), 1.8ml 100mM citric acid-disodium hydrogen phosphate buffer pH7.2 (McIlvaine, 1921 cited by Dawson *et al*, 1969). To this solution was added 200µl of the potato protein fraction and 100µl (0.7 enzyme units) trypsin (Sigma) the enzyme and inhibitor being incubated together for 30min at 30°C prior to addition. The trypsin activity was monitored spectrophotometrically at 410nm due to the liberation of p-nitroanilide groups and the change in absorbance converted to moles min^{-1} using the molar extinction coefficient of p-nitroanilide ($E = 8.8 \times 10^{-3}$ for a 1cm path length). This activity was compared with control reaction tubes containing no tuber protein fractions, the effect of the tuber protein fractions being expressed as percentage inhibition.

Aspects of Applied Biology 33, 1992
Production and protection of potatoes

Amino acids as substrates for the Maillard reaction in stored potato tubers

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INTRODUCTION

The Maillard or non-enzymic browning reaction (Hodge, 1953) is the condensation of reducing sugars and amino acids to produce unacceptably dark fry colours on potato processing. This deterioration has long been solely attributed to increases in tuber reducing sugars during long term storage. In tubers stored at or below 5°C a rapid increase is generally observed, termed low temperature sweetening (Barker, 1932, cited by Burton 1982). This process may be partially reversed by reconditioning tubers at higher temperatures for short periods of up to 6 weeks (Williams & Cobb, 1992). Tubers stored at higher temperatures for prolonged periods exhibit an irreversible increase in reducing sugars known as senescent sweetening (Burton, 1966). Storage temperature thus has a profound affect on reducing sugar status and hence the processing potential of tubers (Burton, 1982). Whilst reducing sugars are important in determining fry colour it is also known that amino acids are involved in Maillard browning (Burton, 1982; Mauron, 1981) and are also important determinants of fry colour (Roe et al, 1990).

Soluble tuber storage proteins, such as patatin in cultivar Kennebec, have been shown to decline during prolonged storage at 4°C (Racusen, 1983), a process that may be similar to that which occurs during seed embryogenesis and germination. Such a breakdown of seed storage proteins has been shown to result in nitrogen flux through the amides glutamine and asparagine (Dilworth & Dure, 1978). These are present to a high level in several major tuber proteins (Suh et al, 1990) and form the largest fraction of the free amino acid pool in all potato cultivars examined (Davies, 1977). In this study the affect of prolonged storage at 5°C and 10°C on the status and metabolism of amino acids in tubers of cultivars Pentland Dell and Record has been investigated over three consecutive storage seasons.

MATERIALS AND METHODS

Tuber storage and sampling procedure

Tubers of the cultivars Pentland Dell and Record were cured for 2 weeks at 15°C following harvest before storage at the Potato Marketing Board Experimental Station, Sutton Bridge, Lincolnshire, U.K. Tubers were stored at 5° and 10°C and 95% r.h. for up to 40 weeks and sampled at regular intervals. On each occasion, five tubers of both cultivars were randomly selected from each storage regime, packed in insulated boxes and returned to Nottingham for extraction and analysis.

Longtitudinal cores (10mm diameter) were removed from the centre of each tuber and divided into three equal sections: basal, median and apical. In all cases, basal sections were used for extraction and measurement of soluble proteins, amino acids and reducing sugars. Each basal core section was weighed in 5ml absolute ethanol, homogenised with 5ml cold pure water (Milli Q reagent grade), and then centrifuged at 3000 rev/min for 5 min. The supernatant was stored on ice and the pellet resuspended in 3ml pure water and recentrifuged as above. After further re-extractions in 3ml and 2ml of water, the pooled supernatants were frozen at -70°C and then freeze-dried prior to analyses.

Determination of amino acid and soluble protein levels

Amino acid concentration was determined by reverse phase h.p.l.c. using a Beckman Gold system with a model 507 autoampler and model 157 fluorescence detector. The autosampler was fitted with a reagent-addition cassette which enabled pre-column derivatisation with OPA (ortho-phthaldialdehyde)-2-merceptoethanol reagent (Lindroth & Mopper, 1979). A 4.6 x 250mm Beckman Ultrasphere ODS column was used with 4.6 x 45mm precolumn. To prepare each sample, 4mg of freeze-dried extract was dissolved in 0.25mM homoserine in 0.05M HC1 and diluted by a factor of 100, so that each sample contained a 2.5μM internal standard. The sample and derivatising reagent were mixed in equal volumes, allowed to react for 90 sec before 20μl was injected onto the column. The amides were separated by gradient elution with the mobile phase compositions (v/v): A; water 72%, sodium propionate buffer pH6.5 20%, acetonitrile 8%. B; water 42%, acetonitrile 30%, methanol 25%, dimethyl sulphoxide 3%. Details of the gradient elution programme are available on request.

Total amino acids were determined by ninhydrin assay (Hart & Cobb, 1986). Four mg of freeze dried extract was dissolved in 10ml pure water and 1ml of this solution mixed with 1.2ml ninhydrin reagent (3.3g ninhydrin, 240ml 2-methoxyethanol, 50ml 4M aq. acetate buffer containing 25g sodium acetate trihydrate and 5ml glacial acetic acid, and 15ml glacial acetic acid made up to 500ml with distilled water) and 0.8ml hydrazine solution (0.052g hydrazine sulphate in 250ml water with one drop of conc. sulphuric acid). The mixture was boiled for 15 min then allowed to cool before 3ml 50% aq. ethanol was added. After 10 min the absorbance at 570mm was read and compared to a glycine standard curve. Soluble proteins were measured using a Coomassie Blue assay (Sedmak & Grossberg, 1977). Freeze-dried extracts were dissolved as above and 1.1ml added to 5ml of Coomassie Blue reagent (100mg BDH Electran PAGE Blue G90 in 50ml 95% (v/v) aq. ethanol to which was added 100ml 85% (v/v) phosphoric acid and made up to 1 litre with distilled water). The mixture was left for 15 min at room temperature and the absorbance measured at 595nm. This was compared to a standard curve for bovine serum albumin (Sigma Fraction V). Reducing sugars were analysed according to Williams & Cobb (1992) and fry colours provided by the Potato Marketing Board from Sutton Bridge Experimental Station.

RESULTS AND DISCUSSION

Whilst each storage season has yielded different data, five general trends are now beginning to emerge. Firstly, tuber amino acids are usually in excess with respect to the reducing sugar pool (Table 1), the bulk of the amino acid pool being made up of the storage amides asparagine and glutamine. At any one time these amides account for between 50% and 90% of the amino acid pool with glutamate and aspartate as the other major contributors, all other amino acids generally account for less than 10%. These findings are

similar to those of Davies (1977) suggesting that although year to year variation in levels takes place, the general composition of the amino acid pool is consistent.

Table 1. Concentration ranges of major metabolites during storage (mg/g fresh weight)

Cultivar Season	Total free amino acids	Soluble proteins	Storage amides	Total reducing sugars
Pentland Dell				
1989-90	7.73-23.20	1.47-5.60	4.32-7.90	0.01-3.30
1990-91	7.64-13.96	0.03-6.69	5.05-9.30	0.11-6.75
1991-92	5.66-20.81	0.51-8.28	5.30-15.07	1.19-5.95
Record				
1989-90	8.46-22.59	1.35-6.96	5.88-9.72	0.34-4.71
1990-91	5.69-23.37	0.53-11.42	5.02-10.92	0.33-4.16
1991-92	8.77-25.02	1.10-10.27	5.93-17.77	0.37-9.00

Secondly, during storage the free amino acid pool rises due to a breakdown of the soluble protein pool (Fig.1). Such a breakdown is consistent with the findings of Racusen (1983). Tubers of the cultivar Kennebec when stored at 4°C showed a greater than 50% reduction in the soluble protein pool size when stored for 219 days. The increase in concentration of free amino acids resulting from protein degradation would suggest that the composition of the amino acid pool reflects that of the tuber storage proteins, and any amino acid metabolism subsequent to their breakdown. Several major tuber proteins, including patatins and the 22kD family of proteins, contain relatively high levels of the storage amides together with aspartate and glutamate (Suh et al, 1990). These amides are not in as high proportions as in the free amino acid pool, suggesting some amino acid interconversion following protein breakdown.

Thirdly, the amides asparagine and glutamine are present at equal levels throughout storage (Fig. 2) in both cultivars, implying that their metabolism is strictly controlled. Work on cotton seed embryogenesis (Dilworth & Dure, 1978) had suggested the flux of nitrogen on protein breakdown from arginine to glutamine and then asparagine. If applied to potato tubers the asparagine pool would be expected to rise relative to glutamine due to the action of asparagine synthetase, this has not been consistently observed in our studies.

Fourthly, reconditioning of tubers at 20°C for up to 6 weeks does not significantly alter the size or composition of the amino acid pool in either cultivar and as a result, the protein pool is also unaffected. This would imply that any improvement in processing potential caused by reconditioning is solely due to the decrease in reducing sugars (Williams & Cobb, 1992).

Fifthly and lastly, fry colours (Agtron values) were similar in trend to reducing sugars, a relationship in most cases not improved by the inclusion of amino acids. Pentland Dell tubers stored at 10°C were an exception in all three seasons studied, with a deterioration in

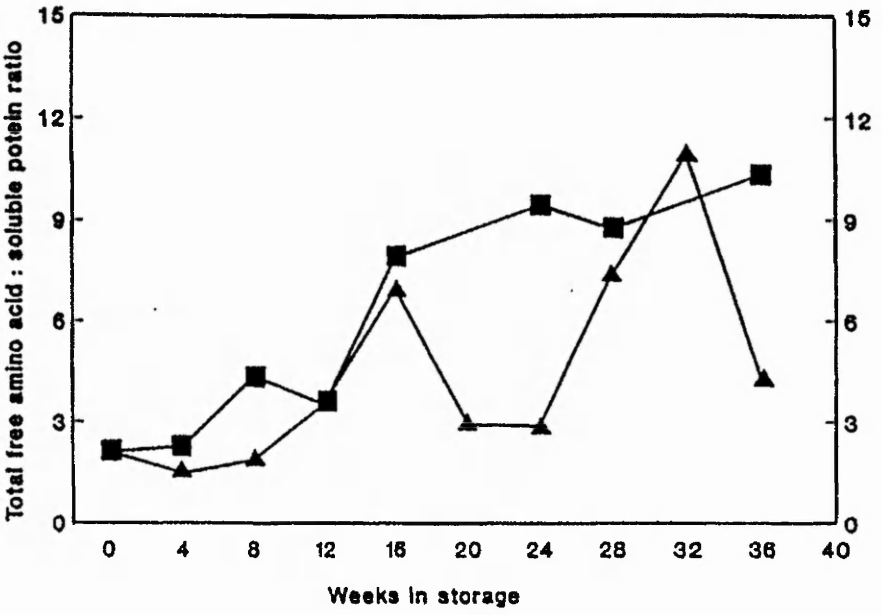


Fig. 1. The ratio of total free amino acids to soluble proteins in Pentland Dell tubers stored at 5°C (■) and 10°C (▲). 1990-91 storage season.

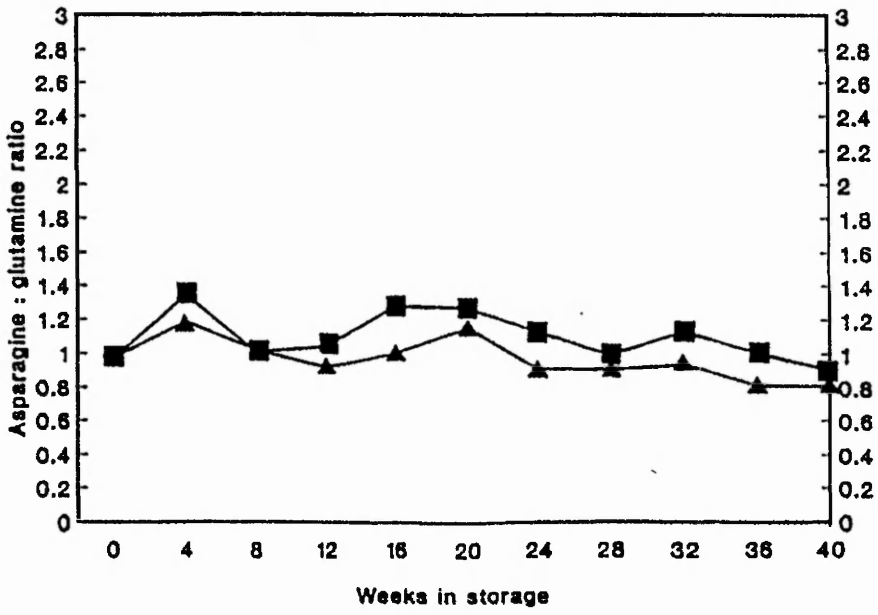
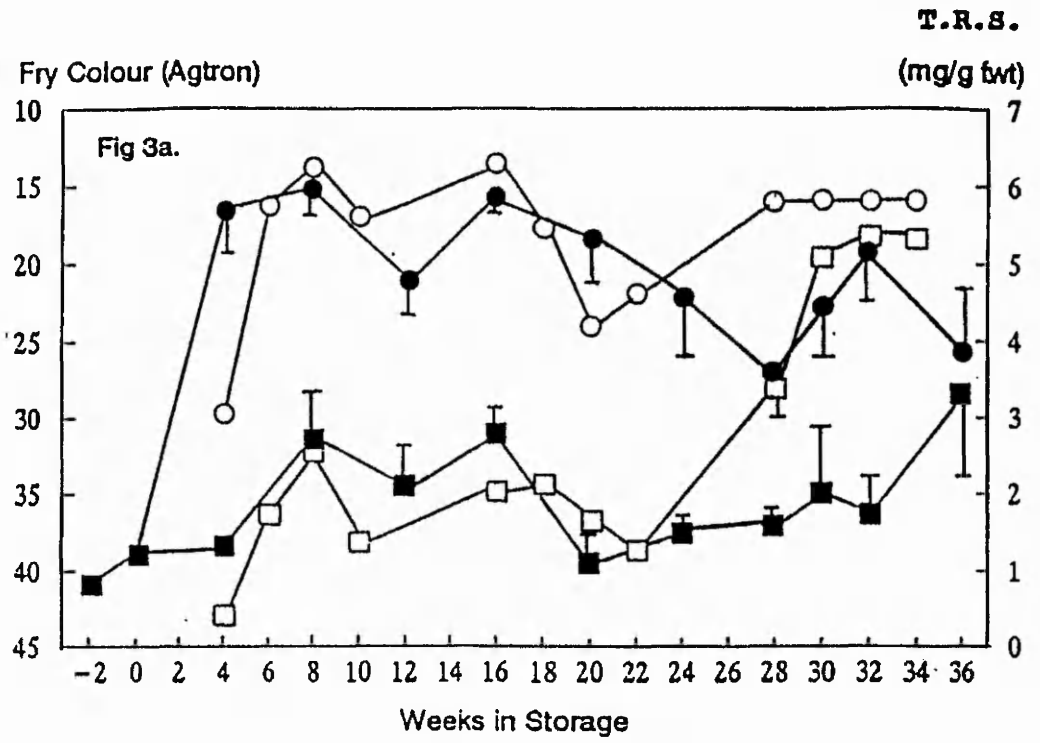
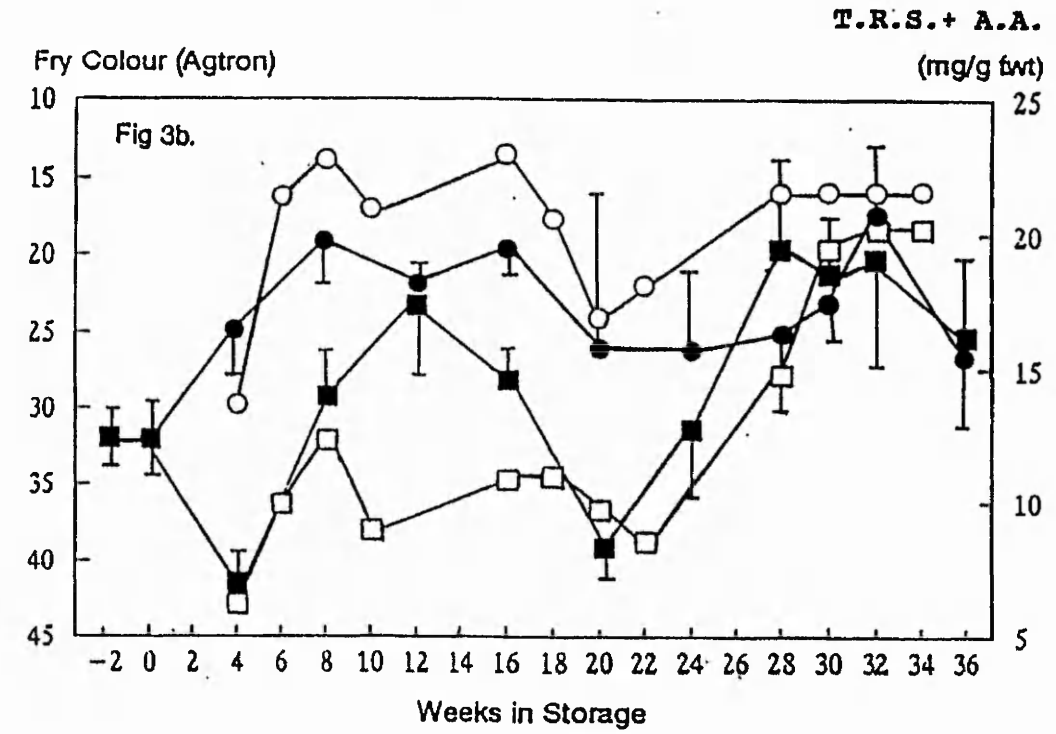


Fig. 2. The ratio of asparagine to glutamine in tubers of Pentland Dell stored at 5°C (■) and 10°C (▲). 1990-91 storage season.





Figs. 3a and 3b. Fry colour by Agtron 5°C (○) and 10°C (□)(a) correlated to: total reducing sugars 5°C (●) and 10°C (■) and (b) the sum of total reducing sugars and amino acids 5°C (●) and 10°C (■). Pentland Dell 1991-92 storage season.

fry colour not being matched by a concurrent rise in reducing sugars. The addition of amino acids to reducing sugar concentrations (Figs. 3a and 3b) improved their similarity with trends in fry colour, the inclusion of individual amides instead of total amino acids did not further improve this. The amino acids although usually in excess with respect to reducing sugars, in these cases may possibly play a synergistic role in colour production (Roe et al. 1990).

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FACTORS AFFECTING PROTEIN TURNOVER IN STORED TUBERS

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Keywords : Storage, protein turnover, proteolytic activity

INTRODUCTION

During prolonged tuber storage it is thought that breakdown of soluble storage proteins occurs (Racusen, 1983) so providing a source of free amino acids to support sprout growth and storage metabolism. Studies by Nowak (1977) have shown that increases of protein may occur during storage. This suggests a turnover of nitrogen rather than a straightforward flux from storage proteins to free amino acids.

In this study, carried out over three consecutive storage seasons, both soluble protein and free amino acid pool sizes have been measured at 5° and 10°C temperature regimes, in order to establish the direction of net nitrogen flow. During the final year of this study the soluble protein and free amino acid pool sizes have been related to proteolytic enzyme activity, a controlling factor of protein breakdown.

MATERIAL AND METHODS

Tubers of the cultivars Pentland Dell and Record were cured for two weeks at 15°C following harvest, and then placed into storage at 5° and 10°C for up to 40 weeks at The Potato Marketing Board Experimental Station, Sutton Bridge, Lincolnshire, U.K. At regular intervals five tubers of each cultivar and temperature were removed from storage and returned to Nottingham for extraction and analysis.

Longtitudinal core samples were taken and the basal sections used for ethanolic extraction of soluble proteins and free amino extracts were freeze-dried and resuspende prior to analysis. Additional basal se were used for extraction and partial purifi of proteolytic enzyme activity durin current year of the study.

Soluble proteins were measured b Coomassie Blue assay (Sedmak & Gros 1977) using bovine serum albumin standard (Sigma fraction V). The tota amino acid pool size was determine ninhydrin assay (Hart *et al*, 1986), individual amino acids were measure reverse-phase HPLC using a Beckman system with C18 column (Brierley & 1992).

Proteolytic enzyme activity was extracte from basal core tissue by homogenisatio pH6 McIlvaine buffer and crude e partially purified by Sephadex G2 filtration. Activity was measured by the n of Twinning (1984) using the breakdo fluorescein isothiocyanate-labelled c resulting in increased fluorescence (excita 490nm, emission λ 525nm).

RESULTS

Results so far obtained have suggested key trends:

Firstly, both breakdown and synthe soluble protein may occur during storag pattern of nitrogen flow varying be season and cultivar. Initial trend proteolytic enzyme activity have sugge key role in determining the pattern of ni

flow, as activity appears correlated to free amino acid concentration.

Secondly, increases in total free amino acids did not always correspond with soluble protein decreases, suggesting that the water insoluble protein pool may also play a role as a source of free amino acids.

Thirdly, in most cases free amino acid and soluble protein pool sizes did not differ between tubers stored at 5° and 10°C, thus implying that temperature has little effect on the direction of net nitrogen flow (Fig 1.).

Fourthly and lastly, initial results have shown no consistent temperature effect on proteolytic activity.

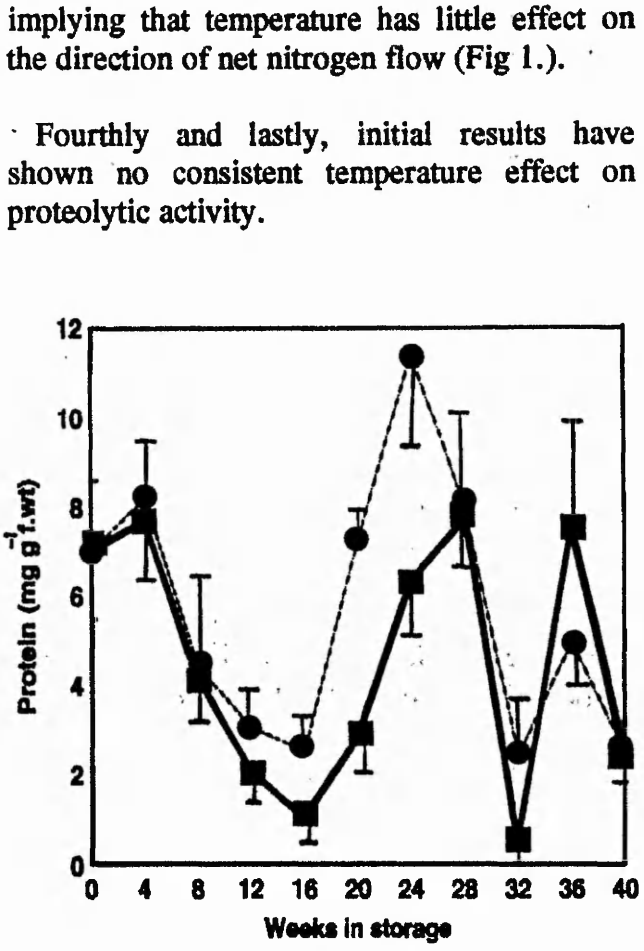

Figure 1. The soluble protein pool of cv. Record tubers stored at : 5°(■) and 10°C (●) during the 1990-91 season.

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