CHARACTERIZATION OF TRYPTIC HYDROLYSATES

OF

PROTEIN ISOLATES

OF

PHASEOLUS BEANS

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CHARACTERIZATION OF PROTEIN ISOLATES OF *PHASEOLUS* BEANS

by

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ABSTRACT

This study was directed at elucidating the structural and functional characteristics of legume protein isolates with different microstructures.

Protein isolates with different microstructures (crystalline and amorphous) were prepared from four varieties of *Phaseolus* beans; white kidney bean and navy bean (*P. vulgaris*), and large lima and baby lima beans (*P lunatus*) The protein isolates were subjected to tryptic hydrolysis at various time intervals, and the effect of hydrolysis on the functional properties of the protein isolates was studied, using bovine casein as control. Reversed phase HPLC with UV detection was used to generate peptide maps of the tryptic hydrolysates, and electrospray mass spectrometry was used to characterize the peptide profile of the protein hydrolysates.

There was no difference between the susceptibility of the crystalline and amorphous protein isolates to tryptic hydrolysis, however, the hydrolytic products of the crystalline isolates showed higher solubility and functional properties compared with those of the amorphous isolates Limited hydrolysis markedly improved the functional properties of both crystalline and amorphous protein isolates. The effect of hydrolysis on the functionality of the bean protein isolates was higher compared with casein. In general the hydrolysates of the bean protein isolates showed higher functional properties than casein and its hydrolysates.

Reversed phase HPLC peptide mapping of the tryptic digests and ESI/MS of the RP-HPLC fractions showed structural and compositional difference between crystalline and amorphous protein isolates prepared from the same bean variety; The results also showed that off-line ESI/MS, and ESI/MS/MS of RP-HPLC fractions of the tryptic hydrolysates could be used for the structural characterization of the protein isolates.

RÉSUMÉ

Cette étude a trait à l'élucidation des caractéristiques fonctionnelles et structurales des isolats de protéines de légumineuses ayant différentes microstructures.

Les isolats de protéines ayant différentes microstructures (cristallines et amorphes) ont été préparées à partir de quatres variétés de fèves *Phaseolus*; fève blanche et fève bleue marine (*P. vulgaris*), grosse et petite fève de lima (*P. vulgaris*). Les isolats de protéines ont été soumis à l'hydrolyse tryptique à différentes intervalles de temps, et l'effet de l'hydrolyse sur les propriétés fonctionnelles des isolats a été étudiée en utilisant de la caséine bovine. La chromatographie liquide à haute performance (HPLC) à phase inversée avec détecteur UV a été utilisée pour générer les mappes de peptides résultant des hydrolyses et la spectrométrie de masse par ionisation électrospray (ESI/MS) a été utilisée pour caractériser le profile.

Aucune différence apparente n'a été observée entre la susceptibilité des isolats de protéines amorphes et cristallines envers l'hydrolyse tryptique, cependant, les produits résultant de l'hydrolyse des isolats cristallines ont montré une solubilité et des propiétés fonctionnelles supérieures à celles des isolats amorphes. L'hydrolyse partielle améliore grandement les propiétés fonctionnelles des deux types d'isolats de protéines. L'effet de l'hydrolyse sur les propiétés fonctionnelles des isolats de protéines de fèves étaient plus marqué avec la caséine. En général leurs produits d'hydrolyses ont montré des propiétés fonctionnelles supérieures à la caséine et ses produits d'hydrolyses.

Les profils peptidiques de digestions tryptiques obtenus par HPLC/ESI/MS en phase inversée ont montré des différences en composition et en structures des isolats de protéines cristallines et amorphes pour une même variété de fève. Les résultats ont aussi démontré que les fractions obtenus et soumises à l'ESI/MS et l'ESI/MS/MS peuvent être utilisés pour la caractérisation structurale isolats de protéines.

DEDICATE TO MY DEAR WIFE

JOYCE IRENE ASHAMI YEBOAH

FOR YOUR LOVE AND CARE

THANK YOU

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Having completed this Masters program, I realize that a number of good people graciously journeyed with me through the good and bad times that I faced in my pursuit, and I would not close the page on this thesis without showing my very sincere gratitude.

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LIST OF ABBREVIATIONS

AMOR (A)	Amorphous Isolate
BLB	Baby Lima Bean
CID	Colission Induced Dessociation
CRYS (C)	Crystalline Isolate
DH	Degree of Hydrolysis
EC	Emulsifying Capacity
ES	Emulsion Stability
ESI	Electrostray Ionization
LLB	Large Lima Bean
LSMEANS	Least Square Means
MS	Mass Spectroscopy
NB	Navy Bean
RP-HPLC	Reversed Phase High Performance Liquid Chromatography
TCA	Trichloroacetic Acid
WKB	White Kidney Bean

I. INTRODUCTION

In recent years a great deal of attention has been paid to the development of new and inexpensive sources of food proteins to meet the growing world demand. Several reports have dealt with plant protein sources particularly legume and cereal proteins. Socio-economic circumstances have also increased the demand for new functional ingredients and the development of fabricated and formulated foods, and natural flavours.

The need to enhance the functional properties of food proteins has lead to the use of both chemical and enzymatic processes to modify their functionality, and to increase their use in food systems. The chemical modification processes that have been used include acylation, acetylation, glycosylation, lipoylation, amidation and deamidation, phosphorylation and dephosphorylation, etc (Feeney et al. 1982). Some of these chemical modification methods can be replaced by non-proteolytic enzyme catalyzed reactions, which have the advantage of milder reaction conditions and a potential for stereospecificity (Whitaker and Puigserver 1982). Chemical and non-proteolytic enzyme modification processes are usually directed at specific functional groups of the side chains of the amino acid residues of the polypeptide chain of proteins. These modification processes result in an increase the net negative charge of the proteins and a decrease of the isoelectric point of the proteins, with a consequent increase in protein solubility.

The most widely used method for food protein modification is proteolysis (Fox et al 1982). Proteolytic modification of food proteins involves the use of a variety of exogenous protease to partially and selectively hydrolyse the food proteins, to produce peptide mixtures with improved functionality over the original proteins. The development of new and improved methods of separation and analysis has also facilitated the production of protein hydrolysates or peptide mixtures with more improved functional properties (Harwalkar and McMahon, 1993). With the increasing demand for food proteins and an increasing trend towards the production and use of improved functional protein ingredients by the food industry, it is important to develop faster and more precise analytical techniques for the evaluation of the nutritional potential of new plant protein sources, and the integrity of formulated or processed foods.

The objectives of this study were to 1). elucidate the structural differences between crystalline and amorphous protein isolates of *Phaseolus vulgaris* (white kidney and navy beans) and *P. lunatus* (baby lima and large lima beans), 2). study the effect of tryptic hydrolysis on the emulsifying and foaming properties of the protein isolates. The study will involve hydrolysis of the protein isolates at various time intervals and evaluating the effect of hydrolysis on the functional properties of the protein isolates. The hydrolysates will also be characterized using reversed phase high performance liquid chromatography and off-line electrospray ionization mass spectrometry. The results of this study may provide information that will increase the present understanding of the differences between crystalline and

amorphous protein isolates with respect to their structure, protein composition and functional properties, and hence the utilization of legume proteins. The study may also provide a protocol for the characterization of food proteins and their hydrolysates using LC-MS techniques.

II. LITERATURE REVIEW

II.A. Utilization of Proteins of Phaseolus Beans.

The common dry beans *Phaseolus vulgaris* (navy, kidney, pinto, and great northern etc.) and *P. lunatus* (the lima beans etc.) are the grain legumes consumed in the greatest quantity in the world (Sathe et al., 1984). Bean proteins are of fair to moderate nutritional quality, and are free of toxic heavy metals and mycotoxin (Satterlee, 1981). The bean proteins are usually prepared by isoelectric precipitation, sieving and/or air clarification, and can be used as food protein fortifiers and extenders (Satterlee, 1981, Chavan and Kadam, 1993). The addition of bean flours and protein isolates of *Phaseolus* beans to wheat flour has been found to increase water absorption capacity, decrease the mixing time, and the stability of the dough (Sathe et al., 1981). Addition of bean flour in amounts above 10% has been reported to be detrimental to dough and bread quality, while the incorporation of bean protein concentrates at the level of 10% on the other hand, produced bread with acceptable quality (Chavan and Kadam, 1993). Finney et al., (1982) have also reported that the use of whole bean flour even at a 5% replacement level produced undesirable off-flavor, while dehulled bean flours at higher (10% - 15%) replacement levels produced bread with acceptable quality. Other workers (Dreher and Patek, 1984; Cady et al., 1987) have demonstrated that the incorporation of *Phaseolus* bean flours in pastry food products increased their protein and mineral content. It has also been reported that legume flours or

protein isolates are well suited for protein enrichment of cereal foods such as bread and cookies (Pomeranz, 1991).

In spite of the high nutritional potential of legume proteins, coupled with the fact that food legumes have served as an integral part of human diet for some 10,000 years (Deshpande, 1993), these proteinaceous plant foods have generally received some negative publicity from food scientists and human nutritionists This has limited their full domestic and industrial utilization The major so called "antinutritional" factors that has limited the full utilization of food legumes includes, their hard to cook nature (Stanley and Aguilera, 1985), low content of sulphur containing amino acids, and the presence of proteinase inhibitors, lectins (haemagglutinins), flatus factors, tannins, phytates, and the low digestibility of their constituent proteins (Deshpande, 1993).

II.B. Digestibility and Nutritional Quality of Storage Proteins

The storage proteins of *Phaseolus* beans constitute about 17.5 to 26% of the dry bean weight (Sathe et al., 1984) The storage proteins consist of 11.52 to 31% albumins (water soluble proteins) and 46 to 81% globulins (salt soluble proteins), indicating that the globulins are the major storage proteins of *Phaseolus* beans. Phaseolin, the major globulin representing more than 50% of the total seed protein of the *Phaseolus* sp. (Deshpande and Damodaran, 1989, 1991a), has been implicated for the low digestibility, and the relatively poor nutritional properties of the bean proteins (Liener and Thompson, 1980; Chang and Satterlee, 1981).

Phaseolin is a 7S trimeric glycoprotein comprising of three different subunits α, β and γ, with 51 - 53, 47 - 48, and 43 - 46 kDa apparent molecular weights respectively (Slightom et al., 1983). Phaseolin displays considerable homology (over 70%) with vicilin (from pea) and β-conglycinin (from soybean), the other 7S storage proteins of food legumes (Deshpande and Damodaran, 1991b). Compared to vicilin and conglycinin, phaseolin is relatively more resistant to both *in vitro* and *in vivo* proteolysis (Deshpande and Damodaran, 1991b). It has been shown that each subunit of phaseolin is composed of two highly structured domains, which are stabilized by hydrophobic domain-domain interaction (Deshpande and Damodaran, 1989). The strong domain-domain interaction of phaseolin has been implicated for its extreme stability to heat denaturation even in the presence of strong denaturants such as sodium dodecyl sulphate (SDS), urea and guanidine hydrochloride (Deshpande and Damodaran, 1990; 1991b). Deshpande and Damodaran, (1989; 1990; 1991b) have demonstrated that the tertiary and quaternary conformation as well as the relative compactness of phaseolin and the other homologous 7S storage proteins are important determinants of their digestibility and relative biological availability. The presence of proteinase inhibitors (Rackis et al, 1985; Li et al., 1989), and haemagglutinins (Liener, 1976; Jaffe and Camejo, 1961) have also been implicated for the relatively poor nutritional quality of legume proteins.

Several other reports have, however, documented that more than 95% of the so-called antinutritional factors of food legumes are usually destroyed under normal cooking conditions. Up to 100% of the proteinase inhibitory activity (Eicher

and Saterlee, 1988), hemagglutinating activity (Paredes-Lopez and Harry, 1989), and the phytates (Estevez and Luh, 1985; Dhurandhar and Chang, 1990) have also been shown to be destroyed during cooking. The resistance of phaseolin and the other 7S legume protein homologues to proteolysis has also been reported to decrease tremendously by thermal denaturation. The improved hydrolytic behaviour of legume proteins after heat denaturation has been attributed to the disruption of their tight, inflexible, and stable tertiary and quaternary structures (Deshpande and Nielsen, 1987; Deshpande and Damodaran, 1989).

The sulphur containing amino acid (cysteine and methionine) deficiency of legume proteins is another reason for which legumes foods are considered nutritionally inferior. However, their high lysine content make them an important source of protein in human nutrition, as lysine is the principal amino acid deficient in many cereal foods (Deshpande and Damodaran, 1990; Deshpande, 1993).

II.C. Characteristics of Proteins With Different Microstructures

Protein isolates from *Phaseolus* beans, having different micro-structure have been shown to exhibit different biological and physical properties. Li et al., (1989) reported that crystalline (bipyramidal) protein isolates from *Phaseolus* beans showed lower trypsin inhibitory (TI) activity than amorphous protein isolates obtained from the same species. They also reported that there was no clear relationship between the TI content and the microstructure of the protein isolates with respect to their susceptibility to tryptic digestion. It was also reported that the

major constituent proteins of *Phaseolus lunatus* were more resistant to tryptic digestion than those of *Phaseolus vulgaris*. Recent work (Di Lollo 1990) has also shown that the crystalline prctein isolates (bipyramidal, and spheroidal) from *Phaseolus* beans show higher solubility and surface hydrophobicity than the amorphous isolates from *Phaseolus* beans.

II.D. Proteolytic Modification of Food Proteins

Proteolytic modification of food proteins with the view of improving their utilization is an ancient art which has been practiced in many parts of the world, particularly in parts of Asia and Africa (Winano, 1979; Hesseltine, 1979), where legumes constitute the primary source of both protein and calories (Deshpande, 1993). Foods which have under gone this type of treatment are referred o as fermented foods. Some common fermented food products are tempeh (soybean fermented by *Rhizopus oligosporus*), Nigerian dawadawa (fermented locust bean) and Chinese sufu (fermented soybean curd) (Winano, 1979; Hesseltine, 1979).

Proteolytic modification of food proteins involve the use of selected peptidases or protease that may be specific or non-specific, to cleave peptide bonds along the polypeptide backbone. This results in the production of molecules (peptides and amino acids) of smaller size than the original protein. Proteolysis also increases the hydrophilicity of protein solutions due to the production of extra amino and carboxyl terminal groups (Phillips and Beuchat, 1981). Proteolysis is also accompanied by a complex series of chemical and physical changes which

can alter protein functionality through sub-unit dissociation, or unfolding of the compact structure of protein to expose their interior hydrophobic regions.

II.E. Effect of Proteolysis on the Nutritional Properties of proteins

From the nutritional point of view, *in vitro* digestion of food proteins before consumption creates favourable conditions for intralumen digestion and increases the availability of protein nitrogen to the consumer (Rebeca et al., 1991). Dietary nitrogen consumed in the form of peptides has been found to be more beneficial than intact proteins or free amino acid mixtures for the following reasons:

a) Small peptides (di and tri peptides) have been reported to have an absorptive advantage over free amino acids mixtures (Rerat et al., 1988.; Rees et al., 1988; Grimble et al., 1987; Grimble et al 1986). This absorptive advantage has been attributed to the nature of the transport systems responsible for amino acid and peptide absorption. The transport system responsible for amino acid absorption has been reported to occur under saturating conditions while the transport system responsible for peptide absorption occurs under non-saturating conditions, respectively. The driving force for the active transport of amino acids is a Na^{+} gradient (Webb et al., 1993). Peptide absorption, on the other hand is believed to be driven by a multiple transport system, namely, a protomotive (H^{+}) force, and possibly a Ca^{+2} gradient (Webb et al., 1992; Webb et al., 1993). Other authors (Ganapathy and Leibach, 1985; Silk et al., 1985; Webb, 1990; Mathews, 1991) have also reviewed the subject of peptide absorption in mammals. Intact

peptide absorption may however be species dependent, as certain animals possess gastrointestinal tracts with unique anatomical features that permit more extensive absorption of peptides. This may be especially true for ruminants, in which extensive microbial fermentation is an essential part of their digestive system (Webb et al., 1993).

b) Peptides have also been reported to afford a more efficient utilization of food protein nitrogen for body protein synthesis (Katsumi and Onodera, 1991).

c) Some peptides of protein hydrolysates have been reported to stimulate hormonal and enzyme secretions, thereby creating a favourable condition for intralumen digestion (Katsumi et al., 1992).

d) Protein hydrolysates provide an alternative nitrogen source as a replacement for whole or intact proteins (particularly milk proteins) in infant formulae designed for infants with food hypersensitivity, or who are allergic to intact protein (Pahud and Schwarz, 1984; Kumar and Hamed, 1991; Sampson et al., 1991). Several investigations (Otani et al., 1990; Cordle et al., 1991; Gmoshinskii et al., 1991) on the use of enzyme hydrolysed food proteins in infant formulae for allergy prone infants have documented that extensive hydrolysis or low molecular weight peptide preparation from protein hydrolysates is essential to render proteins immunologically unreactive. Limited proteolysis on the other hand is required to improve the functional properties of proteins (Turgeon et al., 1992).

Protein hydrolysates or peptide mixtures also play an important role in parenteral nutrition for maintaining the nutritional status of patients who for clinical

reasons cannot feed through the digestive tract and for individuals who cannot digest intact proteins (Cuthbertson, 1950).

The increased number of amino groups resulting from proteolysis also increases the interaction between protein nitrogen and sugars to form maillard type products during food processing. Although this may improve the sensory characteristics of the food product, it also diminishes the bio-availability of protein nitrogen, with a resultant decrease in the nutritional quality of the food product (Harwalkar and McMahon, 1993).

II.F. Effect of Proteolysis on Protein Functionality

The term functionality of proteins refers to any physicochemical characteristic that affects the processing and behaviour of proteins in food systems (Kinsella, 1976). The physicochemical characteristics of proteins can be explained by their hydration, surface and rheological properties. These properties depend on the size and structure (primary, secondary, tertiary and quaternary structures) of the protein molecule which in turn are affected by environmental factors such as pH, ionic strength, solvent polarity and temperature. Several authors (Arai and Fujimaki, 1987; Adler-Nissen et al., 1983, etc) have reviewed the effects of enzymatic hydrolysis on the functional properties of proteins.

II.F.1. Hydration Properties

The ability of proteins to dissolve and disperse in water, absorb and hold water, swell in water or thicken aqueous based systems determines their hydration properties. Solubility is considered to be the most important hydration property of proteins, primarily because of its influence on other functional properties (Nakai and Li-Chan, 1988). The solubility of proteins under a given set of solution conditions depend on their net charge, size, physical and chemical characteristics of their surface and the thermodynamics of their interaction with the surrounding solution (Damodaran, 1989). In solution, the polypeptide chains of proteins tend to fold into systematic secondary conformations, ie., the helices, β-pleated sheets, turns and random coils. Individual polypeptide chains (protein subunits) further associate through covalent (disulphide cross-links) and noncovalent (hydrophobic and van der Waals interactions) in solution to form three dimensional tertiary and quaternary structures in order to attain the minimum internal energy content.

The hydration energy of proteins is due to the interaction between the ionizable functional groups on the protein molecules (ie., the amino and carboxyl termini, and the hydrophilic functional groups of the side chains of basic and acidic amino acid residues) and vicinal water molecules through electrostatic attractions and hydrogen bonding. Gibbs free energy of hydration (δG°_{h}) can be divided into an enthalpic (δH°_{h}) and an entropic ($T\delta S^{\circ}_{h}$) energy terms, as defined in equation [II.1] (Ooi et al., 1987), where δH°_{h} is the enthalpy due to hydration, and δS°_{h} is the energy related to the mobility of dissolved protein molecules. A decrease in the

enthalpy of hydration (exothermic) and an increase in molecular mobility due either to temperature increase or reduction in molecular size (due to hydrolysis) will favour increased protein solubility.

$$\delta G^{\circ}_{h} = \delta H^{\circ}_{h} - T\delta S^{\circ}_{h} \qquad \text{II.1}$$

Proteolytic modification of proteins increases the number of charged groups due to the formation of extra amino and carboxyl termini from the cleavage of peptide bonds of the polypeptide chain. This enhances protein-water interaction through the formation of hydrogen bonds and electrostatic attraction, with a resultant increase in the hydration properties of proteins. The smaller sizes of protein degradation products also increases molecular mobility with a consequent increase in solubility. Several studies (Chobert et al.,1988; Kim et al., 1991; Turgeon et al., 1992; Rebeca et al., 1991) have also reported that proteolytic modification of food proteins increases the protein nitrogen solubility and the zeta potential of protein solutions.

II.F.2. Surface Properties

The amphiphathic nature of proteins allows them to interact with the molecules of both polar and non-polar solvents, and promotes their adsorption at the interface of oil/water and water/air systems to reduce the interfacial tension (Mitchell, 1986; Hung and Zayas, 1991). The surface properties of proteins can be explained by their ability to form and stabilize emulsions, foams and protein/oil

films. The stabilizing effect is achieved through the diffusion of protein molecules from the solution to the interface, where they unfold and spread (Mitchell, 1986) to reduce the interfacial tension. These processes are thermodynamically favourable, as the conformational energy of protein molecules are lost at the interface. It is considered that proteins are adsorbed on oil surfaces through multiple adsorption sites by their hydrophobic regions to form a thin film covering around the oil droplets (Tornberg, 1990; Shimizu et al., 1986). Protein hydrophobicity has been reported to be the major factor affecting their surface activity (Mahmoud et al., 1992). It has been reported that the emulsifying properties of proteins depend on their surface hydrophobicity, while their foaming properties depend on their total protein hydrophobicity (Nakai and Powrie, 1981). A significant linear correlation has been shown to exist between protein hydrophobicity and the interfacial tension decay, and the emulsifying and foaming properties of proteins (Kato, and Nakai, 1980; Townsend and Nakai, 1983; Das and Kinsella, 1990).

Proteolytic modification of food proteins produces smaller molecular weight compounds (peptides and amino acids), which have higher rates of solubility, dispersibility and diffusion than the original proteins, thus providing an entropic driving force towards increased surface activity (Song and Damodaran, 1987). It has been reported that limited proteolysis (24 to 27% non protein nitrogen) of casein and vegetable proteins had very little effect on the surface hydrophobicity of proteins, however, further hydrolysis (40 to 58% non-protein nitrogen) drastically

decreased (10 fold decrease) the surface hydrophobicity (Townsend and Nakai, 1983; Mahmoud et al., 1992). Quaglia and Orban, (1990) have also made similar observations with fish proteins. Mahmoud et al., (1992) argued that, since the surface hydrophobicity of proteins is due mainly to the hydrophobic aromatic amino acid residues (such as tryptophan, phenylalanine, and tyrosine) which usually reside at the surface of proteins due to their bulky structure, enzymatic hydrolysis will increase the surface hydrophobicity of proteins at the early stages of hydrolysis because of the exposure of the interior hydrophobic aliphatic amino acid residues (such as alanine, valine, leucine and isoleucine) which tend to lodge in the interior of the folded protein structure. The observed slight decrease in hydrophobicity at the early stages of hydrolysis has been attributed to the increase in the zeta potential or the net charge frequency (with a concomitant increase in hydrophilicity) which seems to counteract the hydrophobic effect of the exposed hydrophobic amino acid residues (Mahmoud et al., 1992). Increased surface charge due to excessive succinylation has been reported to decrease surface hydrophobicity (Paulson and Tung, 1987).

II.F.2A. Emulsification and Foaming:

The process of emulsification and foaming involves a rapid disruption and coalescence of the dispersed oil globules during the vigorous agitation process. Protein or peptide molecules in the continuous phase adsorb onto newly formed oil droplets and protect them from coalescing (Bergenstahl and Claesson, 1990).

Upon adsorption, proteins or peptides unfold, spread and interact cohesively with each other to form a continuous two dimensional net-work at the interface (Tornberg et al., 1990) ie., a protein lipid film which reduces the interfacial tension and increases the interfacial viscosity, thus preventing coalescence of dispersed droplets. The main factors that affect the emulsifying and foaming properties of proteins and peptides include solubility, rate of diffusion, hydrophilic/hydrophobic balance and molecular flexibility ie., the ability of the protein molecule to under go rapid conformational changes in solution (Turgeon et al., 1992; Tornberg et al., 1990). Emulsifying capacity (EC) measurements can therefore be used as an index to determine the ability of proteins or peptides to protect oil globules from coalescing during emulsification (Dagorn-Scaviner et al., 1987), and as a measure of their rate of adsorption at the interface (Tornberg et al., 1990).

The mechanism involved in the adsorption and emulsification of proteins is different from that of peptides. Proteins are adsorbed on interfaces with trains, loops, and tails (Figure. II.1). The multiple adsorption of proteins at an interface promotes protein unfolding or denaturation, and increases the total free energy released during protein adsorption, and the stability of the adsorbed layer, hence for all practical purposes protein adsorption at the interface can be regarded as irreversible (Tornberg et al., 1990). For rigid or tightly folded protein molecules for which little conformational changes occur during adsorption, adsorption may however be reversible (MacRitchie, 1985). Peptides on the other hand are likely to form a monolayer at the interface by packing closely together.

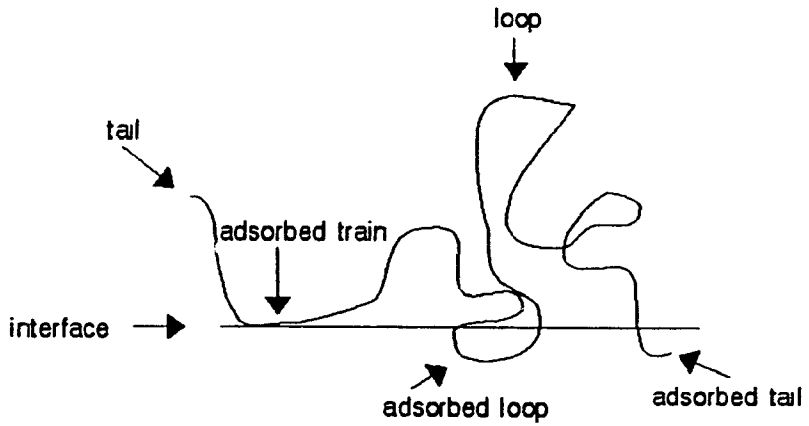

Figure II.1
A schematic conformation of a protein molecule adsorbed at an interface. Protein adsorption at the interface may be due to linear sedgements (train), domains or loops, and terminal sections of the protein that have affinity for the surface.

Although proteins are adsorbed more strongly at interfaces than peptides, the macrostructure of proteins ensures a much slower response to the creation of new surfaces during emulsification or foam formation than smaller molecules (Clark et al. 1987). Proteins also aggregate at fluid surfaces with time and cause the collapse of emulsions and foams (Tornberg and Ediriweera 1987). In a study of the emulsifying properties of two medium sized peptides (hydrophobic and hydrophilic), Lee et. al. (1987) observed that each of the peptides alone had very poor emulsifying activity index (EAI) values, but a mixture of the two peptides improved their emulsifying properties. The improved emulsifying properties of the mixed peptides suggested that there is an interaction between the peptides at the oil/water interface. Unlike proteins which are long and possess multiple adsorption sites, and can form thin film covering around oil globules and stabilize them in oil/water emulsions, smaller peptides have fewer adsorption sites and lack the ability to adsorb tightly at the interface. This leads to a release of the peptide into the aqueous phase (hydrophilic peptides) or into the oil phase (hydrophobic peptides) and causes a collapse of the emulsion.

The presence of a hydrophilic peptide at the water/oil interface enhances the emulsion stability by interacting with the hydrophobic peptide at the oil/water interface to produce an amphipathic complex which has both hydrophilic and hydrophobic moieties. The formation of such complexes at the interface reduces the interfacial tension and stabilizes the surface films. In general small amphiphillic molecules such as peptides are more effective surfactants than proteins, in so far

as they lead to a lower interfacial tension at oil/water interfaces at the same concentration by weight (Dickinson and Wosket, 1989) Proteins on the other hand give rise to thick interfacial films with higher surface viscosity of the dispersed phase once they are absorbed, resulting in a more stable dispersion.

It has been shown that proteolytic modification of food proteins can improve their emulsifying and foaming properties (Puski, 1975, Adler-Nissen, 1982; Chobert et al., 1989; Kim et al..1990) or decrease their emulsifying activity (Deeslie and Cheryan, 1988; Casella and Whitaker, 1990; Quaglia and Orban, 1990; Mahmoud et al., 1992). The functionality of the proteolytic products depend on the starter protein, the type of enzyme used for hydrolysis and the degree of hydrolysis (Haque 1993; Kim et al., 1991). The high molecular weight fractions of protein hydrolysates have been shown to exhibit higher emulsifying and foaming properties compared with the low molecular weight fractions (Turgeon et. al 1992; Ochi ., et al. 1982). Other workers have also shown that a delicate balance between the levels of large polypeptides and low molecular weight peptides is more relevant to the formation and stability of emulsions and foams (Turgeon et al. 1991).

Extensive proteolysis produces low molecular weight peptides which diffuse more rapidly form the bulk of solution to the interface but are less efficient in reducing the surface tension as they are small and have fewer binding sites than proteins, so that they are unable to adsorb strongly and irreversibly at the interface. In general, controlled enzymatic hydrolysis of proteins produces hydrolysates with improved functionality (Adler-Nissen et al., 1983).

II.G. Analysis of Peptides of Protein Hydrolysates

II.G.1. Enzymatic Hydrolysis of Proteins.

The formation of peptides from proteins for the purpose of characterizing the protein require partial hydrolysis using proteolytic enzymes to cleave the polypeptide back bone at specific peptide bonds. The most common enzymes used are the serine proteinases whose catalytic mechanism involves the formation of an enzyme-substrate complex, followed by acylation and deacylation of the enzyme. The specificity of the serine protease is determined by the amino acid residue on the carbonyl side of the peptide bond. Trypsin has affinity for peptide bonds which have lysine or arginine at the carbonyl side of the peptide bond (Trevor, 1991), so that the C-terminal amino acid of tryptic peptides is likely to be either lysine or arginine.

Factors that affect protein hydrolysis include temperature, pH, enzyme concentration, substrate concentration, and the enzyme/substrate ratio. An enzyme substrate ratio of 1:25 w/w or lower has been reported to be adequate for the complete digestion of the protein substrate, while keeping the enzyme level at a minimum to prevent interference due to enzyme autolysis, and promoting enzyme specificity (Stone et al., 1989). Hydrolysis under controlled pH conditions has been reported to solubilize protein nitrogen faster than under uncontrolled pH conditions. Within one hour of hydrolysis of fish meat with *Pescalase* 560, soluble nitrogen reached 67% to 73% under controlled pH conditions, but only 40% to 50% without pH control (Rebeca et al., 1991).

II.G.2. Degree of hydrolysis (DH)

Various studies have shown that in order to obtain desirable properties from protein hydrolysates, the hydrolysis process must be carried out under strictly controlled conditions to a specific degree of hydrolysis (DH) (Adler-Nissen, 1979). DH may be defined as the number of peptide bonds cleaved, as a percentage of the total number peptide bonds of the polypeptide backbone, where the total number of peptide bonds is (n - 1) and "n" is the number of amino acids of the polypeptide (Adler-Nissen, 1986). The amount of peptide bonds cleaved during the hydrolytic process can be assayed by such methods as the formol titration (Taylor, 1957), the trinitrobenzenesulfonic acid (TNBS) (Adler Nissen, 1979), and the ninhydrin reaction method (Clegg et al., 1982). Other methods which measure different changes that occur during the hydrolytic process have also been developed to determine the extent of *in vitro* protein hydrolysis reactions. These include the pepsin-pancreatic digest index (Akeson and Stahmann, 1964) method used for evaluating protein quality, the amount of dialyzable nitrogen from protein hydrolysates calculated as a percentage of the total nitrogen of the hydrolysate (Gauthier et al., 1982; Savoie and Gauthier, 1986). The amount of nitrogen solubilized in trichloroacetic acid (TCA) (Yamashita et al., 1970a; Edward and Shipe, 1978).

Although each of the above mentioned methods is uniquely effective for measuring the different change that occur during protein hydrolysis, none of them is capable of completely describing the total changes that occur during protein

hydrolysis. A combination of these techniques can however provide a comprehensive picture of the *in vitro* hydrolytic processes. A sensitive and accurate assessment of protein hydrolysis is very essential to the food industry for quality control of food protein products and ingredients, for determining protein quality, for food labelling (Nesheim, 1977) and for the critical evaluation of new sources of food protein (Kim and Barbeau, 1991).

II.G.3. Removal of Extraneous Substances From Peptides

Solvent extraction of peptides from food matrices is usually accompanied by the extraction of other undesirable compounds such as proteins, amino acids, amines, sugars and organic acids etc. These extraneous substances interfere with the peptide analysis and need to be removed so as to ease further analysis.

Soluble proteins can be separated from peptides by precipitation. Proteins may be precipitated out of solution by use of protein precipitating agents such as aqueous solutions of TCA, sulfosalicylic acid, and phosphotungstic acid, or with organic solvents such as acetone. Solubilized protein molecules may also be separated from peptides by ultrafiltration techniques, using membranes with specific molecular weight cut off pore sizes, or by size exclusion chromatography.

Amino acids usually co-elute with small peptides and complicate the interpretation of chromatograms, especially when analyzing low molecular weight peptides. Ligand exchange chromatography has been demonstrated to be very effective in separating amino acids from peptides (Monjon and Solms, 1987;

Mojarro-Guerra et al., 1991). In this method, resin in the form of Cu^{+2} complex is used to separate peptides as a group from amino acids. Amino acids form stronger complexes with Cu^{+2} than peptides, as the amino groups of amino acids are more basic, and their carbonyl oxygen is a better electron donor than those of peptides. The stronger Lewis complex formed between amino acids and Cu^{+2} retards their mobility along the column, so that the peptides elute ahead of the amino acids.

Simple sugars, organic acids and salts also interfere with peptide separation. These classes of compounds can be removed by passing the crude peptide solution through solid phase ion exchange or reversed phase cartridges such as the Acell Plus Sep-Pak cartridges (Waters Millipore). This treatment also affords concentration of the peptide sample (Spanier and Edwards, 1987; Voirin and Letavernier, 1991), removes other solvent impurities and can also be used to partition complex peptide solutions into simplified peptide pools with specific chemistries. Ion exchange chromatography and dialysis may also be used to deplete peptide solutions of sugars, organic acids and salts.

II.H. Separation and Characterization of Peptides

Separation techniques based on charge (ion exchange chromatography), size (ultra filtration, gel filtration, or gel permeation and size exclusion chromatography), hydrophobicity (reversed phase, and hydrophobic interaction chromatography), adsorption (paper chromatography, and TLC), electrophoretic mobility and affinity chromatographic techniques etc., have all been used for the

separation of peptides. Although each of these techniques is uniquely effective for the separation of peptides, reversed phase high performance liquid chromatography (HPLC) has emerged as the technique of choice for peptide mapping, the separation and purification of peptides (Stone and Williams, 1986; Stone et al , 1989b). In many cases the purification of peptides from complex mixtures such as protein hydrolysates may require a combination of two or more of the above techniques (Polo et al., 1992; Cobb and Novotny, 1992).

II.H.1. Reversed Phase HPLC

Reversed phase high performance liquid chromatography (RP-HPLC) is primarily based on an adsorption mechanism, which involves a non-polar stationary phase and a polar mobile phase. The stationary phases normally used are the siloxane type alkyl bonded phases containing Si-O-Si-R groups, were -R is the alkyl functional group responsible for the activity of the stationary phase. The length of the alkyl group determines the volume of the organic phase, the sample retention characteristics and the loading capacity of the column (Ravindranath, 1989). The n-alkyl chains mainly used include octadecyl (C_{18}), octyl (C_8), butyl (C_4) and propyl (C_3) chains. More recently, there has been an increasing trend towards the use of polymeric materials such as polystyrene, divinylbenzene and polymer coated silica gels, which are more chemically stable at higher pH values than the bonded phase materials (Swadesh, 1990; Kato et al., 1990). Young and Wheat (1990) have also reported that the use of polymeric supports over wide pH ranges

with inverted ion pair reagents such as alkylamines enhances selectivity of reversed phase HPLC.

Mobile phase systems employed in reversed phase chromatography of peptides are usually made up of two buffer systems: A). An aqueous buffer consisting mainly of water with an ion pairing agent (alkanoic acids, phosphate salts, triflouro acetic acid, etc)., and B) an organic buffer consisting usually of acetonitrile or methanol with an ion pairing agent. Elution may be conducted under isocratic or gradient mode, however, gentle solvent gradient conditions have been found to be optimum for peptide separation by RP-HPLC (David, 1989; Young and Wheat, 1990). The sequential use of one or more selectively strong mobile phases followed by a universally strong solvent has also been shown to enhance resolution and peak capacity (Little et al., 1991). The selectivity of mobile phase systems should be designed in such a way that, late eluting compounds are highly retained. The separation mechanism of reversed phase chromatography is based on an adsorptive interaction between the hydrophobic side chains of the amino acid residues of the analyte (peptides) and the alkyl function of the stationary phase, coupled with ionic and polar interactions of the hydrophilic side chains of the anino acid residues of the peptides with the free silanols (unbonded silanol groups) of the stationary phase (Lemieux and Amiot, 1989) The amino acid composition as well as the peptide chain length also contribute to the retention of the peptides during the separation process (Young and Merion, 1989). Acidic, basic, and hydroxylated peptides are poorly retained on reversed phase columns,

and are hence poorly resolved. The addition of ion pairing agents such as trifouroacetic acid (TFA) to the mobile phase system, in the order of 0.1% concentration has been found to be optimum in improving the chromatographic resolution of acidic and basic peptides (Nagarajan et al., 1989). Ion pairing agents act by interacting with the basic amino acid residues in the peptide, and the N-terminal amino groups of peptides (Guo et al., 1987). Recently, Young and Wheat (1990) reported that the use of HCl as an ion pairing agent is a better alternative to TFA in RP-HPLC peptide mapping, in that the peptides are more strongly retained by the hydrophobic stationary phase, and that the optical clarity of HCl in the mobile phase provides a higher sensitivity in the low UV range for peptide detection.

The elution position of peptides can be predicted, based on their amino acid composition (Brown et al., 1982; Guo et al., 1986a). Guo et al. (1986b) have also demonstrated that under the right elution conditions (100% buffer "A" followed by a gradient of 1% buffer "B"/min at 26°C; where buffer "A" is 0.1% TFA in water and buffer "B" is 0.1% TFA in acetonitrile). A high correlation has been reported to exists between the amino acid composition of a peptide and its retention time. It has also been suggested that individual sequence domains within peptide chains exert their own influence on the peptide retention, and can affect the accuracy of predicted retention (Houghten and De-Graw 1987). Milce et al., (1991) have also developed a group (small amino acid sequences within a peptide chain) retention coefficient methodology for the prediction of peptide retention in reversed phase

liquid chromatography. The advantage of retention time prediction in peptide analysis is that the elution position of peptides of interest can be narrowed down to a small section of the chromatogram, thereby reducing further purification time.

Flow rates used in RP-HPLC for peptide separation varies according to the dimensions of the column, the pore size of the column packing, the nature and the complexity of the peptide mixture to be separated. For narrow bore columns, flow rates in the order of 50 µl/min to 150 µl/min are suitable for sample sizes of up to 25 picomoles for optimum resolution to be achieved, and for analytical columns, flow rates of up to 1500 µl/min is optimal for loading levels of 100 to 250 picomoles (Burgoyne et al., 1989). In general low flow rates yield concentrated peptide fractions and hence avoid sample losses through further concentration (Stone et al., 1989a).

Another important factor in RP-HPLC separation of peptides is the column pore size. Columns with small pore sizes (60 - 100 A°) are suitable for separating small peptides while columns with larger pore sizes; (300 - 500 A°) afford better separations efficiency for high molecular weight peptides (MW > 4kD) (Polo et al., 1992).

II.H.2. Other Separation Techniques

Peptide separation by ion exchange chromatography can be done using cation or anion exchangers. Cation exchangers separate peptides mixtures into basic and neutral peptide pools, while anion exchangers separate peptide mixtures into acidic and neutral peptide pools. The most commonly used stationary phase

materials in ion exchange columns are the polymeric based resins (polystyrene-divinylbezene) which exhibit superior physical and chemical stability over a wide pH range (Ravindranath, 1989). These columns are however not suitable for separating polypeptides due to their high degree of cross-linking and are not amenable to high flow rates. They are therefore used in the separation analysis of low molecular weight peptides and amino acids. Silica based ion exchange columns are more suitable for the separation of polypeptides (Polo et al., 1992). Other exchangers used in ion exchange separations include diethyl-aminoethyl (DEAE) and carboxymethyl (CM) cellulose. The selection of an exchanger, particularly for separating peptides from protein hydrolysates depends on the isoelectric point (pI) of the starter protein. Basic proteins will produce hydrolysates with a high proportion of basic peptides (which will carry net positive charges at low pH values) and will require cation exchangers (Young and Wheat, 1990).

Acetate or citrate buffers may be used for anion exchange separations and tris or phosphate buffers for cation exchange separations. The addition of KCl to phosphate buffers have been found to improve the separation of peptides with similar charge (Cachia et al., 1983).

Strong cation exchange chromatography operating at a pH 3.0 is effective for selectively isolating disulphide bridged tryptic peptides, as peptides differing by as little as +1 charge are easily resolved with strong cation exchange columns (Andrew, 1990). At acid pH (pH < 3), most single chain peptides in tryptic digests will be doubly charged, due to the N-terminal amino group and the extra amino

group on the C-terminal -Lys or -Arg residue. Other single chain peptides may have higher net charge due to the presence of histidine residues, and the presence of internal arginine and lysine residues due to trypsin resistant Lys-Pro and Arg-Pro bonds. Disulphide linked peptides will have net charge of +4 or more at pH 3.0 due to the presence of two or more amino termini and carboxyl terminal -Lys or -Arg residues.

Ion exchange pre-column cartridges can also play important roles in reversed phase separation of peptides, in that they can be used to remove SDS residues from peptide mixtures prior to reversed phase chromatography (Kawasaki and Susuki, 1990). When present in sample solutions, SDS binds strongly to the reverse phase columns, and their strongly acid groups act as an ion exchangers which retards the elution of peptides, while at the same time reducing the hydrophobic interaction sites of the column. In general, ion exchange chromatography is used to compliment reversed phase chromatography (Cachia et al., 1983; Dizdaroglu, 1985).

Size exclusion chromatography and ultra filtration are both separation methods based on the size of the analytes to be separated. Size exclusion is a column chromatographic technique in which larger molecules in the mixture are eluted first, while small molecules are retained longer, whereas ultra filtration is a membrane filtration technique which allows molecules of a specific size range to pass through their pores.

Size exclusion, or gel filtration chromatography has been a major technique

in the analysis of sensory peptides from food samples. It has also been used to separate low molecular weight bitter peptides from cheese (Champion and Stanley, 1982; Lemieux et al., 1989), and peptide flavor compounds from beef broth (Cambero et al., 1992). It is also employed as a sample preparation step to reduce the complexity of peptide mixtures such as protein hydrolysates into less complex peptide pools before subjecting them to reversed phase chromatography (Lemieux and Amiot, 1989; Charbonneau, 1989).

The application of ultra filtration to food peptide analysis has been to partition peptide mixtures into pools of peptides with particular size or molecular weight range with improved functional properties than the original hydrolysate. It has also been used to separate low molecular weight "taste" peptides from cheese (Mojarro-Guerra et al., 1991), and whey protein hydrolysates into high molecular weight peptide fractions with better interfacial and emulsifying properties (Turgeon et al., 1991, 1992), and to produce peptide fractions with antioxidant activity from acid whey (Colbert and Decker, 1991).

Electrophoresis is another prominent separation technique employed in protein and peptide separation that is orthogonal to reversed phase chromatography. It involves the separation of charged ions or neutral molecules that are capable of being ionized in solution, on the basis of their mobility under the influence of externally applied electric field. The mobility of ions depend on their charge, size, shape, the applied electric field, and the viscosity of the medium. In the case of amphoteric molecules such as proteins and peptides, the

net charge on the molecule, which determines its mobility can be altered by changing the pH of the electrolyte. Different variations of the technique have been used in the separation of mixtures of proteins and peptides, and in the generation of peptide maps of protein hydrolysates.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) has been used mainly to evaluate changes in protein profile during proteolytic changes in food proteins (Spanier et al., 1988). Although attempts have been made to use this technique in peptide separation (Bothe, 1985), overlapping peptide banding patterns of low molecular weight peptides does not encourage its use in the analysis of low molecular weight food peptides (Spanier et al., 1988).

Capillary zone electrophoresis (CZE) is another electrophoretic separation technique which is based on the charge to size ratio of peptides, and it is gaining ground as a technique in peptide analysis (Wu et al., 1992; Amankwa and Kuhr, 1992). Cobb and Novotny, 1989 have also shown it to be sensitive and have used it to generate peptide maps from 2 ng sample containing 80 fmol of β-casein. They have also used it to selectively determine arginine and tyrosine containing peptides through pre-column derivatization of these amino acid residues in the peptides (Cobb and Novotny, 1992). Peptide migration rates and separation are usually optimized by pH adjustment, and by the addition of micelle forming surfactants such as sodium dodecyl sulphate, or inclusion forming compounds such as the cyclo-dextrins to the buffer system. The latter method of optimization is termed electrokinetic capillary chromatography (EKC) (Lin et al., 1990). The addition of

micelle forming agents to the mobile phase causes a dynamic partition mechanism of the solute to be established, allowing such subtle differences such as size, shape, and hydrophobicity between analyte molecules to be explored to achieve optimum separation.

The separation of peptides based on differences in their isoelectric point, ie., capillary isoelectric focusing (CIEF) has also proved adequate for peptide separation and mapping (Mazzeo et al., 1993). Its resolving power has been shown to be high enough to differentiate between the tryptic digests of closely related proteins such as chicken, bovine, and horse cytochrome C through peptide mapping (Mazzeo et al., 1993). The main limitation to this technique is that, UV detection can only be done at 280 nm, since the ampholytes used to generate the pH gradient absorb below 280 nm. This indicates that only peptides containing tryptophan and tyrosine can be analyzed by CIEF and the detection sensitivity is low. The technique may however be useful for generating abbreviated peptide maps of complex peptide mixtures such as protein hydrolysates for protein identification.

II.I.1. Detection of Peptides

Analyte detection during separation is one of the major factors that determine the success of an analysis. Most contemporary analytical methods require working with minute quantities of samples (at the picomole and femtomole range), so that highly sensitive detection systems, able to detect and quantify

analytes at such levels have become imperative.

Peptide samples at concentration levels higher than 100 ng/ml can easily be detected and quantified accurately by UV detection between 200 nm and 220 nm. The sensitivity of peptide detection at such low UV wave lengths is greatly impaired since most common sample impurities (amino acids, organic acids amines, etc), and mobile phase systems used in peptide separation also absorb at these wave lengths. It is therefore important to carefully clean up samples prior to separation if detection is to be done at low UV wave lengths. Peptides containing aromatic amino acid residues (Phe, Tyr and Trp) can also be detected at 254 nm and at 280 nm due to the absorption of the aromatic ring systems on the side chains of these amino acids.

Because of the difficulties associated with the detection of peptides in the low ultra violet region (200 - 220 nm), peptides can be derivatized with chemical reagents to produce chromophoric compounds that can permit detection at higher and more specific wave lengths with no interference from impurities. The main derivatizing agents that have been used include 1-dimethylaminonaphthalene-5-sulphonyl chloride or dansyl chloride (DNS-Cl), phenylisothiocyanate (PITC), dimethylaminoazobenzene sulphonyl chloride (DABS-Cl), and 9-fluoromethyl chloroformate (FMOC).

Dansyl chloride reacts with primary and secondary amino groups of peptides and amino acids under alkaline conditions to give dansylated derivatives that can be detected at 254 nm. In addition to the desired dansyl amino acid derivative,

other reaction by products such as dansyl sulphonic acid and dansyl amide are formed during the reaction between peptides or amino acids and DNS-Cl. These reaction by products affect the quantitation of the desired product. Tapuhi et al., (1981) have shown that the use of Li_2CO_3 rather than Na_2CO_3 at pH 9.5, and a reagent analyte ratio between 5:1 to 10:1 in darkness is an adequate reaction condition for dansylation Grego and Hearn, (1983) have also reported that pre-column dansylation does not interfere with reversed phase HPLC, but provides a higher detection sensitivity of N-terminal analysis of peptides.

Phenylisothiocyanate (PITC) is another reagent that reacts with primary and secondary amino groups of peptides and amino acids to form chromophoric compounds. The use of PITC as a derivatization agent has been thoroughly reviewed by Cohen and Strydom, (1988) and Polo et al., (1992). PITC has been used extensively for both pre-column and post-column derivatization analysis of peptides, however its main application has been for peptide sequencing by Edman degradation. Several workers (Bidlingmeyer et al., 1984; Elkin and Wusynczuk, 1988) have demonstrated that pre-column derivatization with PITC affords higher detection sensitivity than post-column derivatization with PITC. Pre-column derivatization has also been used to generate peptide maps, and the PTC derivatives of the separated peptides can be directly sequenced by Edman degradation (Colilla et al., 1991). Pre-column derivatization with PITC has also been shown to enhance reversed phase HPLC separation of closely related compounds such as α- and β- isomers of 3-N-oxylyl-2-3-diaminopropanoic acid

(ODAP), a neurotoxin present in seeds of *L. sativus*, while OPA, DAN-Cl, and FMOC-Cl derivatization followed by RP-PHLC separation could not differentiate between the two isomers (Khan et al., 1993)

UV-Visible diode array detectors allow continuous,and simultaneous spectral acquisition at multiple wave lengths and thus permits analysts to manipulate chromatographic spectral data to discern subtle structural details about a compound (Hillier, 1986). Although the absorption bands of aromatic amino acids overlap in the UV range, they exhibt characteristic second-order derivative spectral minima within +-2 nm at 257, 280, and 290 nm for Phe, Tyr and Trp residues respectively, with detection limits of about 10 pmole (10 ng/ml). The use of microbore columns instead of analytical columns can improve detection by about four fold (Grego, et al., 1986; Nyberg et al., 1986).

Peptides containing free α- and ε- amino groups can be selectively derivatized either at the per-column (Boppana et al., 1991) or post-column (Schlabach, 1983; Kucera and Umagat, 1983) stage of analysis with several fluorogenic reagents under basic conditions (pH > 9) to enhance their RP-HPLC separation with high detection sensitivity. At pH < 8 5, ε-amino groups have poor reactivity with fluorescamine, so that only the N-terminal amino groups can react to an appreciable extent. The fluorescent compound formed can be detected at an exitation wave length of 350 nm and an emission wave length of 425 nm. This allows the detection of N-terminally blocked peptides and prevents abortive attempts of sequencing N-terminally blocked peptides by Edman degradation (Schlabach, 1983). Proline containing peptides which resist direct derivatization

can be detected with OPA after an initial oxidative decarboxylation step using hypochlorites (Schlabach, 1983) or with fluorescamine after an initial oxidative decarboxylation step using chlorosuccinimide (Bohlen and Mellet, 1979).

Ortho-phthalaldehyde (OPA) reacts with primary amino groups of peptides and amino acids in the presence of a thiol compound (2- mecapto ethanol) to form an N-subtituted 1-alkyl thio isoindole derivative which is highly fluorescent, and can be detected in the pico mole range at 340 nm, and as low as 50 fmole level using fluorescence detection (Jones and Gilligan, 1983). OPA does not fluoresce on its own, so that the unreacted reagent does not interfere with the analysis. Pre-column OPA derivatization of peptides with PR-HPLC has also been used to generate peptide maps from tryptic hydrolysates (Mendez et al., 1985). OPA derivatization has also been reported to give highly reproducible and precise results, with resolution and sensitivity comparable to dansyl chloride (Mendez et al., 1985). The isoindole derivatives are however not very stable and undergo time dependent degradation. This problem can be overcome by using Naphthalene-2,3-dicarboxaldehyde (NDA) (Montigny et al., 1987) in place of OPA. NDA reacts with the α-amino groups of peptides in the presence of a cyanide ion to form an intense fluorescent N-subtituted 1-cyanobenzyl isoindole (CBI) derivative which permit the detection of peptides at levels less than 200 fmole (exitation 246 nm) or 3 pmole (exitation 420 nm). Another problem associated with OPA application to peptide analysis stems from fluorescence quenching of their peptide derivatives (Allison et al., 1984).

Derivatization of specific amino acid residues having reactive functional groups with fluoregenic reagents can be used to generate abbreviated peptide maps, thus reducing the complexity and difficulty of inteprating chromatograms for the identification proteins. Cobb and Novotny, (1992) have used derivatization of arginine and tyrosin with benzoin and 4-methoxy-1,2-phenylenediamine respectively using laser induced fluorescence detection for the selective determination of peptides containing those amino acids. 1,2-diamino-4,5-dimethoxy benzene has also beenused to selectively determine tyrosin containing peptides at the low pico mole level (Ishida et al., 1986).

Derivatization of the C-terminal carboxyl groups using fluorescent tags such as 9-anthryldiazo methanol and bromomethoxy coumarin have been shown to allow sub picomole detection, however the technique suffers from poor yeild due to the formation of zwitterions betwwen the N-terminal amino and C-terminal carboxyl groups (Yoshida et al., 1985).

The eletrochemical activity of chemical derivatives of peptides has also been exploited for peptide detection. Warner and Weber, (1989) discorvered that the copper II complex of peptides, based on the biuret reaction could be used to determine peptides electrochemically. The reaction involves the complexation of peptides with Cu^{+2} in a basic medium in the presence of tartarate to stabilize the Cu^{+2} ion in solution. The complex formed is electro active and can be oxidized to the corresponding Cu^{+3} (Warner and Weber, 1991). This technique has been used for both post-column (Tsai and Weber, 1990) and pre-column (Tsai and Weber,

1991) detection of peptides. The Biuret reaction based elactrochemical detection is particularly useful for the determination of peptides that lack N-terminal amino groups Due to blockage, and basic amino acid residues (Warner and Weber, 1989). Unlike other amino specific reagents such as fluorescamine, OPA, etc the Biuret method is peptide specific and affords detection limits in the range of 0.2 pmole in a 20 ul injection volumes (Tsai and Weber, 1991). Schlabach, (1984) attempted the biuret reaction technique in an absorption scheme for the detection of peptides and proteins in serum but the sesitivity was poor.

The electrochemical activity of Cyanobenzene isoindole (CBI) derivatives (Nussbaum et al., 1992), NDA derivatives (Oates and Jorgensen, 1989; Nussbaum et al., 1992) and OPA derivatives (Allison et al., 1984) of amino acids and peptides have also been explored for their detection and determination. The electrochemical activity of 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) derivatives of hydroxyproline (Welch et al., 1993) can also be used for the selective determination of hydroxproline containing peptides at the femto mole level, with a signal to noice ratio in the order of 20:1 (Welch et al., 1993).

Electrochemical probes able to respond to thiol and disulfide containing peptides have also been developed for the detection of sulfur containing peptides (Sun et al., 1991).

II.I.2. Mass Spetrometric Detection

Mass spectrometric analysis and detection of proteins and peptides involves the ionization of (protonation, deprotonation, sodiation etc.,) the analyte molecules followed by the mass analysisof the ionized molecules on the bases of their charge to mass ratio. The development of the so called soft ionization methods· Laser desorption (Karas and Hillenkamp, 1988), Plasma desorption (PD) , Fast atom bombardment (FAB) (Barber et al ,1981), and the ion spray techniques; eg., Electrospray (ESI) etc., have opened up several possibilities for the application of mass spectrometry as an analytical tool in the analysis of non-volatile and thermally labile macro-biomolecules such as proteins and peptides. The prime characteristics of these ionization methods is their ability to produce protonated or deprotonated pseudomolecular ion-beams with low internal vibronic energies from polar or ionic compounds in solution, so that little or no fragmentation processes occur during the ionization process and mass analysis. The development of liquid chromatography / mass spectroscopy intrerfaces which allows the direct coupling of liquid chromatographic and capillary electrophoretic techniques to mass spectrometers for on-line mass detection has further improved the use of mass spectrometry in labile biomolecular ananlysis.

In the recent past, interface techniques such as thermospray (Blakely and Vestal, 1983), electrospray (Loo et al., 1989), continuous flow FAB (Caprioli et al., 1986), an aerosole based coupling device (Cappiello and Bruner, 1993) and a particle beam coupling device (Willoughby and Browner, 1984) among others have

been used for on-line detection of peptides. Direct coupling of liquid chromatography with mass spectrometry improves the efficiency of analysis by reducing sample handling losses and analysis time. On-line analysis also allows for selective ion monitoring of the column effluent for individual peptides. The main constraint imposed on coupling is the high column effluent flow rate compared with the solvent removal capacity of the vacuum system in mass analyzers (Frenz, et al., 1990). This constraint can be overcome by post column splitting of the column effluent to reduce the amount of solvent entering the mass spectrometer (Covey et al., 1991), or by using microbore and capillary columns that tolerate the low flow rates amenable to continuous flow interfacing (Moseley et al., 1991; Cappiello and Bruner, 1993) of liquid chromatography with mass spectrometry. Micro-bore HPLC also permits highly efficient separation with minimal solvent consumption. Even with the low sample capacity, micro-bore HPLC has proved to be indespensible to liquid chromatography / mass spectrometric analysis (Cappiello and Bruner, 1993).

On or off-line peptide detection with mass spectroscopy is independent of chromatographic resolution, in that co-eluting peptides from chromatographic separations are further resolved into individual peptide molecular ions by the mass analyzer. On-line mass spectroscopic detection has also been used to follow proteolytic reactions in immobilized enzyme reactors (van Breemen and Davis, 1992).

Electrospray ionization involves an electrically induced nebulization process that produces highly charged droplets, which are then shrank to their size limit in

dry nitrogen at atmospheric pressure (60°C).This process results in the desorption of intact, multiply charged molecular ions. The multiple charging phenomenon of ESI extends the effective molecular weight range of conventional mass spectrometers by a factor equal to the number of charges, and the potential for obtaining structural information on the molecular ions (Loo et al., 1989). The ionization of proteins and peptides in aqueous solutions (pH < = 4) is due to protonation of the amino groups of the basic amino acid residues (arginine, lysine, and histidine) and the N-terminal amino group. Negatively charged molecular ions formed as a result of deprotonation of protein and peptide molecules may also be produced by electrospray ionization under basic (pH > 11) solution conditions (Loo et al., 1992) Tryptic peptides usually desorb as doubly charged molecular ions $(M + 2H)^{+2}$ due to the extra amino groups of lysine of arginine at the C-terminal of tryptic peptides (Hail et al., 1990). Tryptic peptides containing histidine residues may also show triply charged $(M + 3H)^{+3}$ molecular ions in addition to the doubly charged molecular ion (Covey et al., 1991).

Peptides containing disulfide bonds can also be identified from tryptic digests by mass spectrosmetric detection, since such peptides exhibit more intense molecular ion peaks of +3 and +4 charged states over the doubly charged state, due to the additional basic residue of Lysine and Arginine and the amino termini (Covey et al , 1991). Mass spectrometry can also be used for the specific and selective detection of Glycopeptides (Huddleston et al., 1993). In this tecnique, signals derived from nonglycosylated peptides are virtually eliminated giving an ion

current chromatographic trace of only glycosylated peptides. the technique is also able to distinguish between N- or O- linkages with all common oligisaccharides (Huddleston et al., 1993).

Collision induced dissociation of mass selected peptide ions followed by the mass analysis of the fragments, can be used to characterize peptides through partial or tota' sequencing following daughter ion mass detection (Biemann, 1986). The fragmentation process usually produce mass peaks that correspond to fragment ions formed by the cleavage of -CO-NH- bond along the peptide chain. This cleavage partern results in the formation of an extensive series of ions which can be used to generate the structure of the parent molecular ion (Figure IV.24). The ion series that usually dominate the CID spectra are the a_n, b_n, and y_n ion series. a_n, and b_n ion types generally dominate if a basic amino acid is lacated at or near the N-terminus or if there is no basic amino acid residue in the peptide, and y_n ion types dominate if a basic amino acid is located at the C-terminus (Johnson et al., 1987). It will therefore be expected that y_n ion series would dominate the CID mass spectrum of tryptic peptides. Cleavage at the β, γ bonds of the side chains of amino acid residues result in additional ion series (w_n ion type) which can be used to differentiate closely related amino acids such as leucine and isoleucine (Johnson et al., 1987). A complete identification of an ion series can provide a complete sequence information of an unknown peptide. Mass sequence analysis can also be utilized to determine the position of a disulfide bridge in proteins (Bean and Carr, 1992). Both on-line and off-line LC/MS/MS

analysis of peptide mixtures (tryptic digests) provide a relatively simple and elegant means of obtaining optimum structural information with minimum sample consumption (Covey et al., 1991).

III. MATERIALS & METHODS

III.A. Source of Beans Samples and Materials.

Samples of dried white kidney beans and navy beans (*Phaseolus vulgaris*), and large lima and baby lima beans (*Phaseolus lunatus*) were obtained commercially from a local store. The dried beans were ground in a Micro sample mill (Pulverizing machinery Ltd., Summit, NJ). The powdered samples were stored in air tight plastic containers at room temperature (23 - 25°) until ready for use. Bovine casein, bovine pancreatic trypsin, and reagent grade (ACS approved) citric acid were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals were reagent grade from BDH (St.Laurent Que.). Distilled deionized water (Millipore Ultra pure) was used throughout the study.

III.B.1. Citric Acid Extraction:

Citric acid soluble proteins with bipyramidal crystalline micro-structures were isolated from each of the four varieties of *Phaseolus* beans according to the procedure of Melnychyn (1969). The finely ground bean flour were suspended in citric acid solutions (1 L.) with appropriate concentrations (normality) and pH (Table III.1.) to make 10% w/v suspensions and stirred occasionally for 1 hr at room temperature. The resulting mixtures were centrifuged at 4000g for 10 min. The supernatant was filtered through glass wool and stored at 5° for 18 hrs to crystallize out the extracted protein. The micro-structure of the precipitated protein isolates were verified under a light microscope. The protein isolates were

recovered by centrifugation at 4000g for 10 min. After centrifugation the residue was washed twice with distilled deionized water and lyophilized.

III.B.2. Sodium Hydroxide Extraction:

Isoelectric precipitated proteins were extracted from ground bean flour of the four varieties of *Phaseolus* beans according to the method of Fan and Sosulski (1974). The ground beans were suspended in aqueous sodium hydroxide solutions (0.02%) to make 10% w/v powder suspensions at room temperature. The suspensions were allowed to stand for 1 hr with intermittent stirring After an hour, the mixtures were centrifuged and the supernatant filtered through glass wool, and the residue discarded. The pH of the supernatant was adjusted to 4.5 with 10% HCl to precipitate the dissolved proteins. The protein isolates were recovered by centrifugation (4000g for 10 min). The recovered protein isolates were washed twice with distilled deionized water and then lyophilized. The microstructure of the proteins were verified under a light microscope.

III.C. Determination of Protein Content

The protein nitrogen content of the protein isolates, and the total nitrogen extracted from the powdered bean were determined by the automatic Kjeldahl method (A.O.A.C., 1985) using the Labconco Rapid Still III Kjeldahl system (Labconco Co-op. Kansas City, MO.). All determinations were done in duplicates.

Table III.1. Conditions for Extracting Crystalline Protein Isolates

Phaseolus Beans	Conc(N) / pH of Citric Acid
Navy Bean:	0.1 / 5.5
White Kidney Bean:	0.5 / 4.5
Large Lima Bean:	0.08 / 3.5
Baby Lima Bean:	0.08 / 3.5

III.C.1. Total Nitrogen Extracted From Bean Flour

To determine the total nitrogen extracted from the powdered beans, quantities (10 ml) of the supernatant obtained after the initial centrifugation of the protein extract solutions (after filtering through the glass wool) were taken and placed into 250 ml Rapid Still III digestion tubes One tablet of kjeltab (catalyst composed of 3.5g K_2SO_4 and 175 mg HgO; Fisher Scientific Ltd. Montreal Que.), 10ml concentrated H_2SO_4, and 3 drops of anti-foam were added. The tubes and their content were placed in the Labconco rapid "digester-4" at 410°C and digested for 45 min. After digestion the tubes were allowed to cool and 55 ml of water were manually added to the contents of the tubes to prevent crystallization of ammonium sulfate. The tubes were then placed in the Labconco distillation unit, and 80 ml of Noah:$Na_2SO_3.10H_2O$ (50%:5%) were dispensed automatically into the digestion tubes and distilled The liberated ammonia were trapped in 50 ml of 4% boric acid solution containing 3 drops of methyl red/bromocresol green indicator. The amount of liberated ammonia trapped in the boric acid solution was determined by titrating with 0.05 N HCl. A conversion factor of 6.25 was used to calculate the protein content.

III.C.2. Protein Nitrogen Content of Isolates

To analyze the protein nitrogen content of the protein isolates, 0.25 g of the lyophilized protein isolates were dissolved in distilled deionized water and the pH adjusted to 8, to make a final volume of 5 ml. 5 ml of 20% aqueous trichloro acetic

acid solution was then added to the resulting protein solution to precipitate the true protein nitrogen. The precipitated protein were recovered by centrifugation (5000g, 20 min). The protein nitrogen content of the residue was determined by the automatic Kjeldahl method.

III.D. Tryptic Hydrolysis

Quantities of the lyophilized protein isolates and casein corresponding to 1.6g protein nitrogen (as determined by the Kjeldahl method) were suspended in water (50 ml) and the pH of the suspension was adjusted to 8.0 by the dropwise addition of 10% NaOH. The volumes of the protein solutions were then made up to 100 ml with distilled deionized water and incubated at 38° in a water bath. Trypsin solutions were also prepared by dissolving 0.064 gm of trypsin in 50 ml of water and adjusting the pH to 8.0. with 10% NaOH. The volume of the trypsin solution was finally made up to 100 ml with distilled deionized water and incubated at 38°. The protein and trypsin solutions were mixed together to make a 200 ml solutions with final protein concentration of 0.8% w/v, and an enzyme substrate ratio of 1:25. The hydrolysis mixtures were incubated at 38° in a reciprocal shaker water bath throughout the hydrolysis experiment, with constant shaking (90 rpm). Samples of the hydrolysis mixtures were withdrawn at time intervals of 0, 5, 15, 30, 45, 90, and 180 min of hydrolysis to determine the degree of hydrolysis (DH).

III.D.1. TCA Solubility Index

To determine the percentage of protein nitrogen solubilized in trichloroacetic acid (TCA) by tryptic hydrolysis, quantities (10 ml) of the hydrolysis mixtures were withdrawn at time intervals of 0, 15, 30, 45, 90 and 180 min and immediately added to 10 ml of 20% cold trifluoroacetic acid solutions to precipitate the unhydrolyzed protein on very high molecular weight polypeptides. The resulting suspensions were centrifuged (5000g for 10 min) to separate the precipitated protein nitrogen from the solubilized nitrogen. After centrifugation the nitrogen content of the supernatant solutions were determined by the micro Kjeldahl method. The amount of protein solubilized or nitrogen solubility index (NSI) was calculated from equation [III.1] (Deeslie and Cheryan, 1981)

$$\text{NSI} = \frac{\text{TCA solubie nitrogen in hydrolysate}}{\text{Total nitrogen in hydrolysate}} \times 100\% \qquad \text{[III.1]}$$

III.D.2. Determination of Degree of hydrolysis (DH)

Quantities (10 ml) of the hydrolysis mixture were withdrawn at time intervals of 0, 5, 15, 30, 45, 90, and 180 min of hydrolysis and the hydrolysis reaction terminated immediately by heating rapidly to boiling. The hydrolyzate solutions were allowed to cool to room temperature, and 10 ml of 15% neutralized formaldehyde (pH, 8.5) was added and titrated with 0.05N NaOH using phenolphthalein as indicator to an end point pH of 8.5. The hydrolysis mixtures at zero time of hydrolysis (unhydrolyzed protein solutions) were used as the blank or control (Taylor, 1957). The amount of α-amino acid nitrogen (AN) liberated as

determined by the direct formol titration method was calculated from the following relationship: 1 ml of 0.1 N NaOH = 1.4 mg liberated α-amino nitrogen.

III.D.3. Statistical Analysis:

The data obtained for the degree of hydrolysis experiment (as determined by Formol titration), and the nitrogen solubility experiment were analyzed statistically by the analysis of covariance in a randomized complete block design (Steel and Torrie, 1980).

The effects of time of hydrolysis (as the regression component of the statistical model), bean variety, and extraction treatment (ie., citric acid extraction or crystalline protein isolates, and Noah extraction or amorphous protein isolates) as the variance component of the model, were analyzed as the Log_e(time of hydrolysis) X 4 levels of bean varieties X 2 levels of extraction treatment.

Differences in the variables studied were estimated as differences in their Least Mean Squares (LSMEANS), and the T-test was used to test for significance.

III.E. Functional Properties of Protein Hydrolysates:

III.E.1.Emulsifying Properties.

To determine the emulsifying properties of the protein hydrolysates and the effect of hydrolysis on the emulsifying properties of the bean protein isolates, quantities (5 ml) of the hydrolysis mixtures were withdrawn at time intervals of 0, 5, 15, 30, 45, 90, and 180 min of hydrolysis and the hydrolysis reaction terminated

immediately by heating rapidly to boiling. The pH of the hydrolysate solutions were adjusted to 7.0, and the volume made up to 30 ml with distilled deionized water. The emulsions were prepared by adding corn oil (Mazola oil, 10 ml) to the 30 ml hydrolysate solutions and homogenizing for 10 min, using a Virtis homogenizer at a speed of 70. After emulsification, the emulsions were transferred into 50 ml test tubes and allowed to stand for 12 hrs at room temperature. The emulsions separated into two phases, an upper water in oil emulsion and a lower oil in water emulsion. The oil volume fraction (ϕ) of the oil in water emulsions was determined by specific gravity measurements using the following equation [III.2].

$$\phi = (\rho_{aq} - \rho_{em})/(\rho_{aq} - \rho_{oil}) \qquad \text{III.2}$$

where ρ_{aq} is the specific gravity of water, ρ_{em} is the specific gravity of the emulsion and ρ_{oil} is the specific gravity of oil (Klemaszewski et al., 1992).

The surface area of the emulsion and the mean radius of the dispersed oil droplets were determined by a modification of the method of Pearse and Kinsella (1978). Quantities (1 ml) of the lower oil in water emulsions was taken and diluted (50 to 400 fold) with 0.2% sodium dodecyl sulfate (SDS) solution to give an absorbance reading between 0.2 to 0.7 at 550 nm. The surface area and the mean droplet radius ($\bar{R}$) were calculated from equations [III.3] and [III.4] respectively.

$$\textbf{Surface Area} = (4.606 * A)/(\phi * L) \qquad \textbf{III.3.}$$

$$\bar{R} = 3\phi/\textbf{Surface Area} \qquad \textbf{III.4.}$$

III.E.2. Foaming Properties

To study the effects of hydrolysis on the foaming properties of the protein isolates, samples (20 ml) of the hydrolysis mixtures were drawn at 0, 15, 30, 45, 90, and 180 min intervals of hydrolysis, and the reaction terminated by heating rapidly to boiling. The pH of the hydrolysates were then adjusted to 7.0 with NaOH (10%) or HCl (10%), and transferred into 50 ml graduated cylinders, and the volumes were made up to 20 ml with distilled water. Foam was developed by agitating the hydrolysate solutions in the measuring cylinders at a speed of 15,000 rpm for 2 min using a polytron (PT-MR 3000, Kinematica AG, Brinkmann, Littau, Switzerland). The total volume was noted (V_o), and the foam expansion (FE %) (Britten and Lavoie, 1992) was calculated from the following equation [III.5].

$$FE(\%) = (V_o / 20) \times 100 \qquad \text{III.5}$$

The serum holding capacity of the foams stabilized by the protein hydrolysates were determined by measuring the volume of serum draining from the laminar structure of the foams, at 2, 5, 15, 15, 20, 25, and 30 min intervals.

Foam stability (FS) were determined as a function of the foam volume (V_{30}) remaining after 30 min of foam formation, according to equation [III.6]. It has been found that 30 min drainage is required to reach maximum stiffness (Britten and Lavoie, 1990).

$$FS(\%) = 100 - [(V_o - V_{30}) / (V_o - 20)] \times 100 \qquad \text{III.6}$$

III.F. Analysis of Hydrolysates by RP-HPLC

Tryptic peptides produced during the hydrolytic process were separated by RP-HPLC and detected by UV, using a diode array detector to generate peptide maps, and resolve the hydrolysates into individual peptide components for further analysis. The chromatographic spectral data were acquired with the wave scan software (version 1.04 LKB, Sweden) and integration of spectral data was done with the Nelson Analytical software package (version 1.4, 1980) (Perkin-Elmer Oak Brook, Illinois). All HPLC separations were run under the following conditions:

Analytical column:	C_{18} LKB Ultro-Pac column (4 mm x 250 mm)
Solvent system: in 1 hr	Dual solvent system under gradient elution (10% B to 50% B Solvent A = 0.1% TFA in distilled deionized water. Solvent B = 0.09% TFA in acetonitrile (BDH Inc).
Flow rate:	1 ml/min: Maximum pressure = 250 bar.
Sample size:	20 µl injection volume.
Guard column:	Brownlee 3 cm C_{18} cartridge column
Detector:	LKB diode array UV (model 2140).
HPLC equipment:	LKB HPLC dual pump system (model 2150).

III.F.1. Sample Preparation for Direct RP-HPLC

Samples for RP-HPLC separation were prepared by taking quantities (15 ml) of the hydrolysis mixtures at 0, 5, 15, 30, 45, 90, and 180 min intervals of hydrolysis, and terminating the hydrolysis reaction by heating rapidly to boiling. The hydrolysates were then filtered through 0.1 μm millex filter membranes (Millipore Waters, St. Louis, MO.) to remove any particulate substance. Quantities (20 μl) of the filtrates were then injected for HPLC analysis.

III.F.2. Sample Preparation Using Solid Phase Extraction Cartridges

To improve on reversed phase HPLC separation of the protein hydrolyzates, the total hydrolysates were first partitioned into acidic, basic, and neutral peptides peptide pools using the Sep-Pak Plus solid phase extraction cartridges (Waters Chromatography Division, Millipore Co-op, Milford, MA.). The Sep-Pak Plus cartridges; accell plus CM (cation cartridge; to retain basic peptides), accell plus QMA (anion cartridge; to retain acidic peptides), and the C_{18} (reversed phase cartridge; to retain neutral peptides) cartridges were first conditioned with appropriate solvents and connected in series in that order with teflon tubing as illustrated in figure III.1. The cartridge arrangement will reduce sample loss through leaching.

Samples to be loaded on the cartridges were prepared by taking quantities (5 ml) of the filtered hydrolyzates as prepared in Section III.E.1. and adjusting the pH to 7 0 by dropwise addition of 1% NaOH or HCl. The neutral hydrolysates were then loaded on the assembled cartridges at the QMA end as show in figure III.1.

The samples were allowed to sip into the upper cartridge by gravity. After the sample had completely passed through the upper cartridge, distilled deionized water (10 ml) was introduced into the cartridge system and allowed to elute by gravity. This eluent containing unwanted, weakly bound components were discarded. The loaded cartridges were then disconnected from each other, and the desired components eluted from the cartridges with appropriate buffers (Figure. III.2). The acidic peptides bound on the QMA cartridge were eluted with a NaCl buffer (5%, pH 4), the basic peptides bound on the CM cartridge were eluted with NaCl buffer (5%, pH 8.5), and the neutral peptides bound on the C_{18} cartridge were eluted with 90% methanol. Peptide fractions obtained from the ion exchange cartridges were further passed through C_{18} cartridges to remove the buffer salt. The final eluent fractions obtained from the C_{18} cartridges were concentrated under a stream of dry nitrogen, and 20 μl aliquotes were taken and analyzed by reversed phase-HPLC.

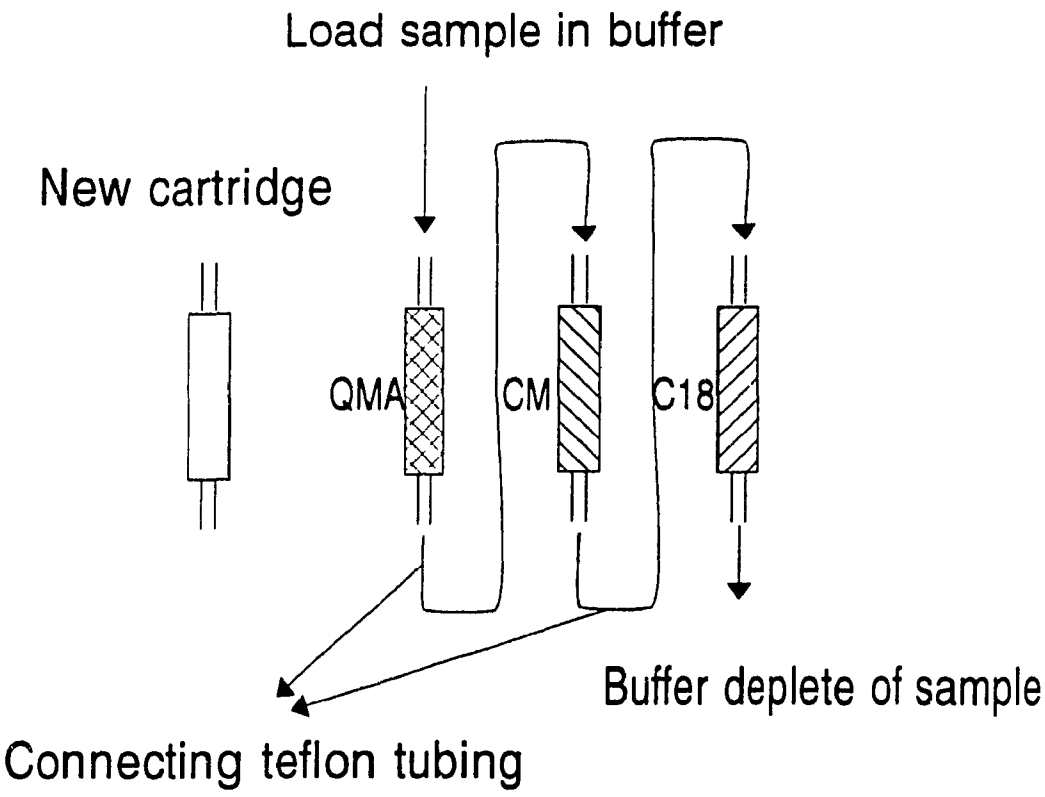

Figure III.1
The arrangment of solid phase extraction cartridges used to partition protein hydrolysates into acidic, basic and neutral peptide pools. QMA (anion exchanger) retains acidic peptides; CM (cation exchanger) retains basic peptide and C-18 cartridge retains neutral hydrophobic peptides. The new cartridge is first conditioned by passing distilled deionized water through it.

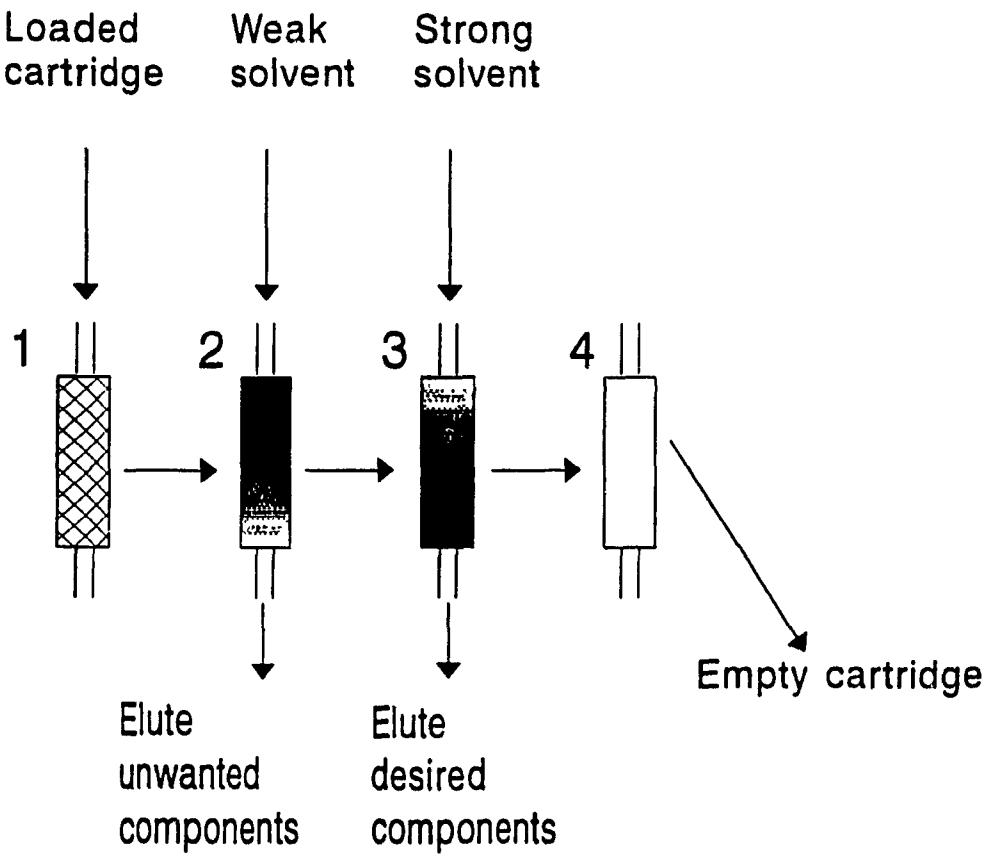

Figure III. 2
A schematic showing the elution protocol of adsorbed peptide molecules from the solid phase extraction cartridges. 2). The loaded cartridge is first eluted with a weak buffer (distilled deionnized water), to remove salts and weakly bound extraneous material; 3). the desired, strongly bound components are the eluted with a strong buffer (methanol, NaCl solutions with varing pH depending on the chemistry of the bound peptides)

III.F.3. Sample Preparation for Mass Spectrometry

Fractions collected from reversed phase HPLC separation of the protein hydrolysates were first evaporated to dryness in a Speed-vac system. The dried samples were redissolved in 70 μl of freshly prepared aqueous acetic acid (10%) solution just before introduction into the mass analyzer. The acid conditions under which the samples are introduced into the electro-spray ion source generates molecular ions in the positive ion $(M + nH)^{n}$ mode. The maximum charged state of molecular ions depends on the number (n) of amino groups available for protonation.

III.G. ELECTRO-SPRAY MASS SPECTROMETRY

A triple quadrupole mass spectrometer (API III MS/MS system, SCIEX, Thornhill, Ontario, Canada) was used for all mass analysis and partial sequence analysis of peptides. The instrument has a mass to charge ratio (m/z) range of 0 - 2400, and permits the selection of the ^{12}C mono-isotopic specie of the molecular ion isotopic cluster. Data acquisition and analysis was handled by a MacIntosh IIx computer. Samples were infused into an electro-spray ion source (fused silica capillary of 100 μm ID) of the mass spectrometer at a rate of 1 μl/min from a 50 μl capacity micro syringe mounted on a low pressure infusion pump (Model 22, Harvard Apparatus, South Natick, MA). The electric potential of the capillary orifice was set at +30 V for mass calibration and molecular weight (MW) determination (ESI/MS), and was raised to +100 V for partial sequence (ESI/MS/MS) analysis. For molecular weight determination, the first rod set Q1 (-40 V) of the triple

quadrupole system was used to select the appropriate molecular ion, while the other rod sets; Q_2 and Q_3 of the triple quadrupole system served as ion guidance lenses. For partial sequence analysis, Q1 (-70 V) was used to select precursor ions, Q2 (radio frequency only quadrupole, -70 V) was used as the collision chamber (Helium as collision gas), and Q3 (-70 V) was used to resolve daughter ions. Figure III.3 shows a schematic diagram of the API III MS/MS system. The mass spectrometer mass to charge ratio scale was calibrated with lysozyme in 10% acetic acid at an infusion rate of 1 µl/min. To minimize errors introduced at the calibration stage, an alternate calibration protocol was adopted by using the multi-channel averaging (MCA) function provided by the instrument control software (Feng et al., 1991).

III.G.2. Identification of Tryptic Fragments of Phaseolin

To identify the tryptic peptides of phaseolin that may be present in the hydrolysates, the m/z ratios of theoretically generated tryptic fragments $(M + H)^+$ of α, and β phaseolin were compared with singly charged molecular ions $(M + H)^+$ of the ESI-mass spectra of the RP-HPLC fractions of the hydrolysates. Since different peptides may have the same molecular weight and m/z ratio for their protonated molecular ions $(M + H)^+$, the integrity of identified tryptic fragments of phaseolin verified by MS/MS partial sequence analysis. See appendix A.3 and A.4 for all possible tryptic fragments of α- and β- phaseolin respectively.

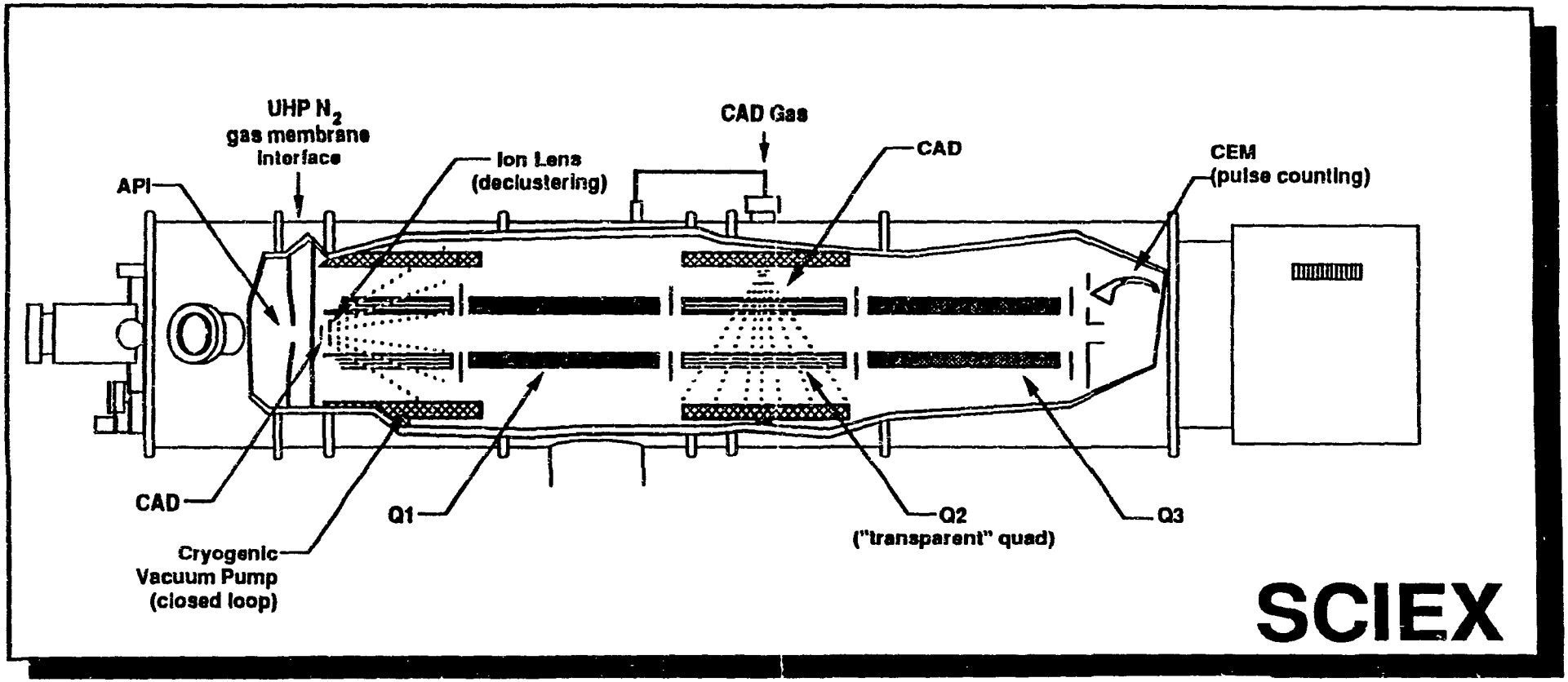

Figure III.3. Schematic diagram of the SCIEX Atmospheric Pressure induced Ionization Triple Quadrupole MS/MS system (API III).

IV. RESULTS AND DISCUSSION

IV.A.1. Protein Solubilized from Powdered Beans

Table IV.1 shows the amount of nitrogen solubilized from the powdered beans by citric acid solutions and dilute NaOH, expressed as percentage of the dry weight of the powdered beans. The results show that dilute NaOH is more effective in solubilizing protein nitrogen from the powdered beans than citric acid solutions. Dilute NaOH solubilized protein nitrogen corresponding to 17.6% to 19.9% (> 86% of total seed nitrogen) of the dry bean weight, while the citric acid solutions solubilized protein nitrogen corresponding to 8.4% to 13.15% (< 56% of total seed nitrogen)of dry bean weight. The protein nitrogen content of *Phaseolus* beans ranges from 18.12% to 23% (Deshpande et al., 1981). The lower solubility of the seed proteins in the citric acid solutions compared with dilute NaOH can be attributed to the initial pH's of the citric acid solutions (3.5 to 5.5) which were in the region of the isoelectric points (pH of 4 to 5) of the seed globulins (Sathe et al., 1984). It is also possible that the pH of the citric acid solution will altogether exclude the extraction of those seed proteins whose isoelectric points were at the pH of the citric acid solutions. The storage proteins of *Phaseolus* beans may be classified under two groups, ie.. the albumins (water soluble proteins) which make up 11.52% to 31% of the total crude proteins, and the globulins (salt soluble proteins) which make up 46.2% to 81% of the crude proteins (Sathe et al., 1984). It is also possible that the citric acid extracted protein isolates may contain a higher

proportion of albumins than the dilute NaOH extracts since the albumins are more soluble in the isoelectric point region than the globulins.

Table IV.1. Total Protein Nitrogen Solubilized from Bean Flours Under Citric acid and Sodium Hydroxide Extraction Conditions

	Citric Acid (% of Dry Bean)	Dilute NaOH (% of Dry Bean)
Navy Bean:	8.40	18.70
White Kidney Bean:	13.15	19.70
Large Lima Bean:	12.32	19.90
Baby Lima Bean:	8.90	17.60

Table IV.2. Protein Nitrogen Content of Crystalline and Amorphous Isolates

	Crystalline Isolates	Amorphous Isolates
Navy Bean:	93.86%	75.48%
White Kidney Bean:	94.33%	72.87%
Large Lima Bean:	89.24%	73.22%
Baby Lima Bean:	91.08%	81.45%

Note: The values are averages of duplicate runs

IV.A.2. Protein Content of Isolates

The crystalline protein isolates generally showed higher protein content (ranging from 89.24% for BLB to 94.33% for WKB) than the amorphous isolates (ranging from 72.87% for WKB to 81.45% for BLB) (Table IV.2). The higher protein content of the crystalline isolates can be explained by the nature of crystallization and precipitation of the crystalline and amorphous protein isolates respectively. The slow and systematic nature by which the crystalline protein isolates were crystallized out of the citric acid solutions ensures a more homogeneous aggregation of protein molecules with little entrapment of extraneous substances. By comparison, the amorphous protein isolates formed by isoelectric precipitation results from a rapid process of precipitation of protein molecules from solution, which will more likely be accompanied by the entrapment of extraneous material, thereby reducing the purity of the amorphous protein isolates.

IV.B.1. Degree of Hydrolysis (Formol Titration)

The degree of hydrolysis (DH) determined as the amount of amino nitrogen (amino-N) liberated during hydrolysis is related to the extent of peptide bond cleavage of the polypeptide backbone of proteins (Mahmoud et al., 1992), and the conformational characteristics (particularly the susceptibility of the polypeptide chains of the proteins to enzymatic hydrolysis) of the protein (Sathe et al., 1984). Figures IV.1 and IV.2 show the results of the degree of hydrolysis for the crystalline and amorphous protein isolates respectively. The results show that the

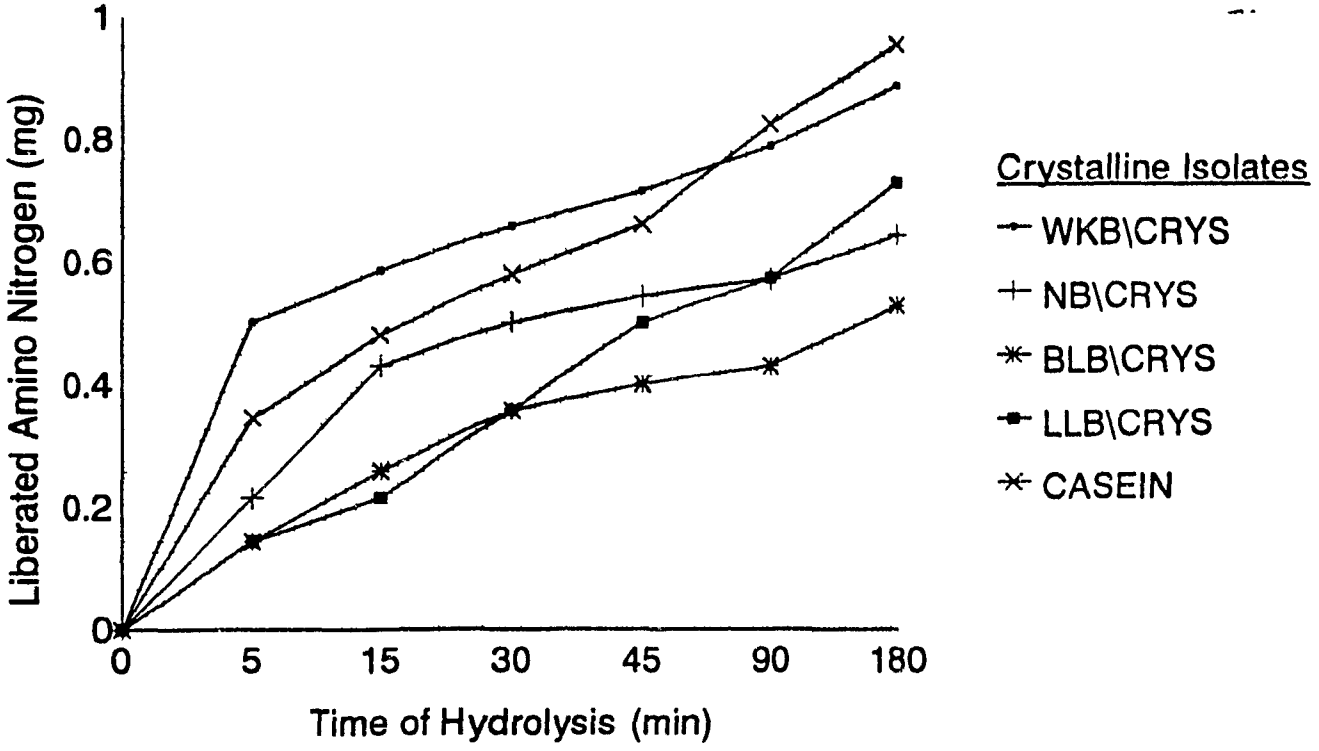

Figure IV.1
A graph of the amount of amino nitrogen (mg/g protein) liberated from the crystalline isolates and casein by tryptic hydrolysis with time, as determined by Formol titration

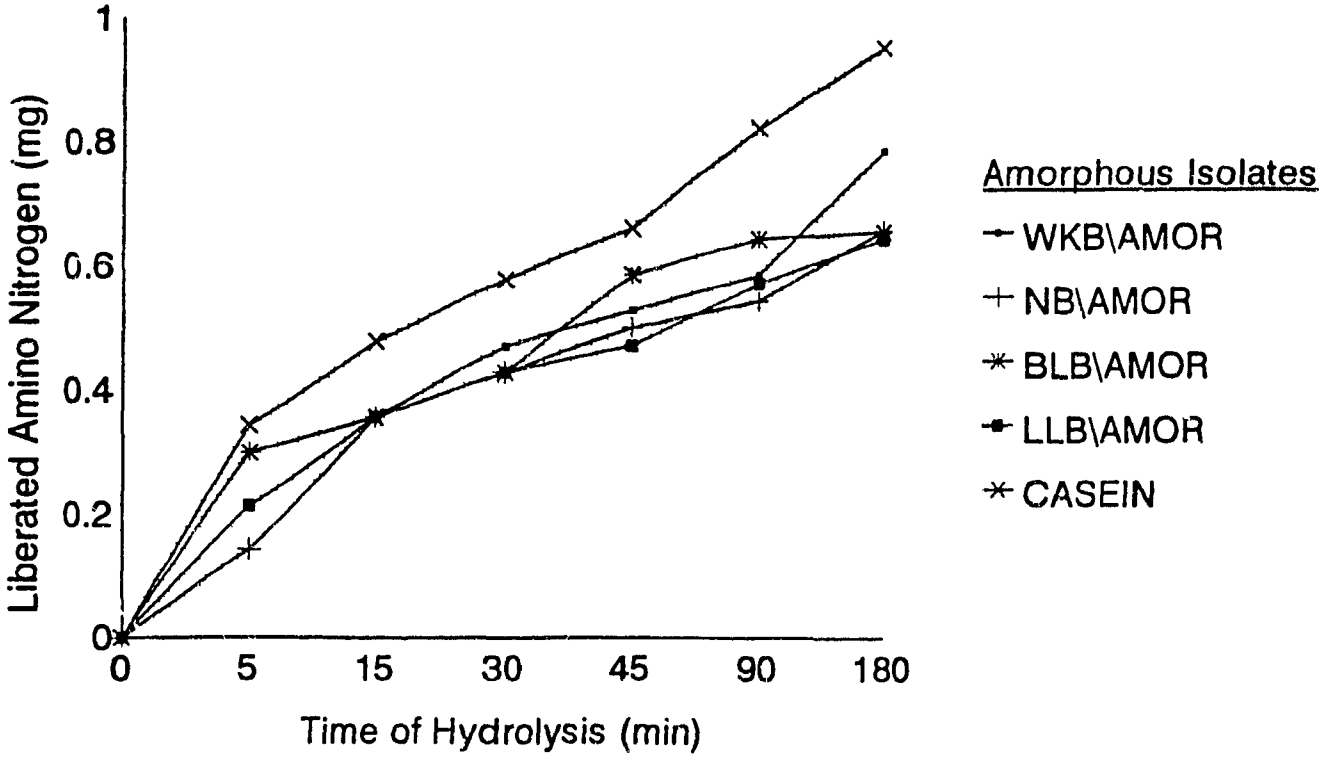

Figure IV.2
A graph of the amount of amino nitrogen (mg/g protein) liberated from the amorphous isolates and casein by tryptic hydrolysis with time, as determined by Formol titration

hydrolysis curves followed a typical enzyme catalyzed reaction progress (Whitaker, 1972). With the exception of the crystalline protein isolate of WKB, casein showed a higher initial rate of hydrolysis compared with the bean protein isolates. The exceptionally high initial rate of tryptic hydrolysis of the crystalline protein isolate of WKB suggests that their trypsin susceptible peptide bonds are more exposed to the enzyme in the native form of the protein isolate compared with the trypsin susceptible peptide bonds of casein and the other bean protein isolates. The results also show that there was a greater disparity among the crystalline protein isolates with respect to tryptic hydrolysis (Fig. IV.1) than there was among the amorphous protein isolates.

The results of the statistical analysis (See Appendix, Table A.1 to A5) show that the least squares means (LSMEANS) difference of amino-N liberated, between the crystalline and amorphous protein isolates of NB (0.046 mg/gm protein., $P < 0.2$) and LLB (0.029 mg/ gm protein., $P < 0.3$) were not significant. The LSMEANS difference of amino-N liberated between the crystalline and amorphous protein isolates of WKB (0.197 mg/ gm protein., $P < 0.001$), and BLB (-0.13 mg/ gm protein., $P < 0.001$) were however highly significant. The results show that there is no clear relationship between the microstructures of protein isolates of *Phaseolus* beans with respect to their susceptibility to tryptic hydrolysis (Fig. IV.3).

Compared with casein, the bean protein isolates were less susceptible to tryptic hydrolysis (Figures. IV.2 and IV.3). The lower degree (DH) of the bean protein isolates compared with casein could be due to the presence of trypsin

inhibitory factors (Rackis et al., 1985; Li et al., 1989) in the bean isolates. The more compact native structure of the major globulins of the bean protein isolates (Chang and Satterlee, 1981; Deshpande and Damodaran, 1990, 1991b) could also be implicated for their lower digestibility.

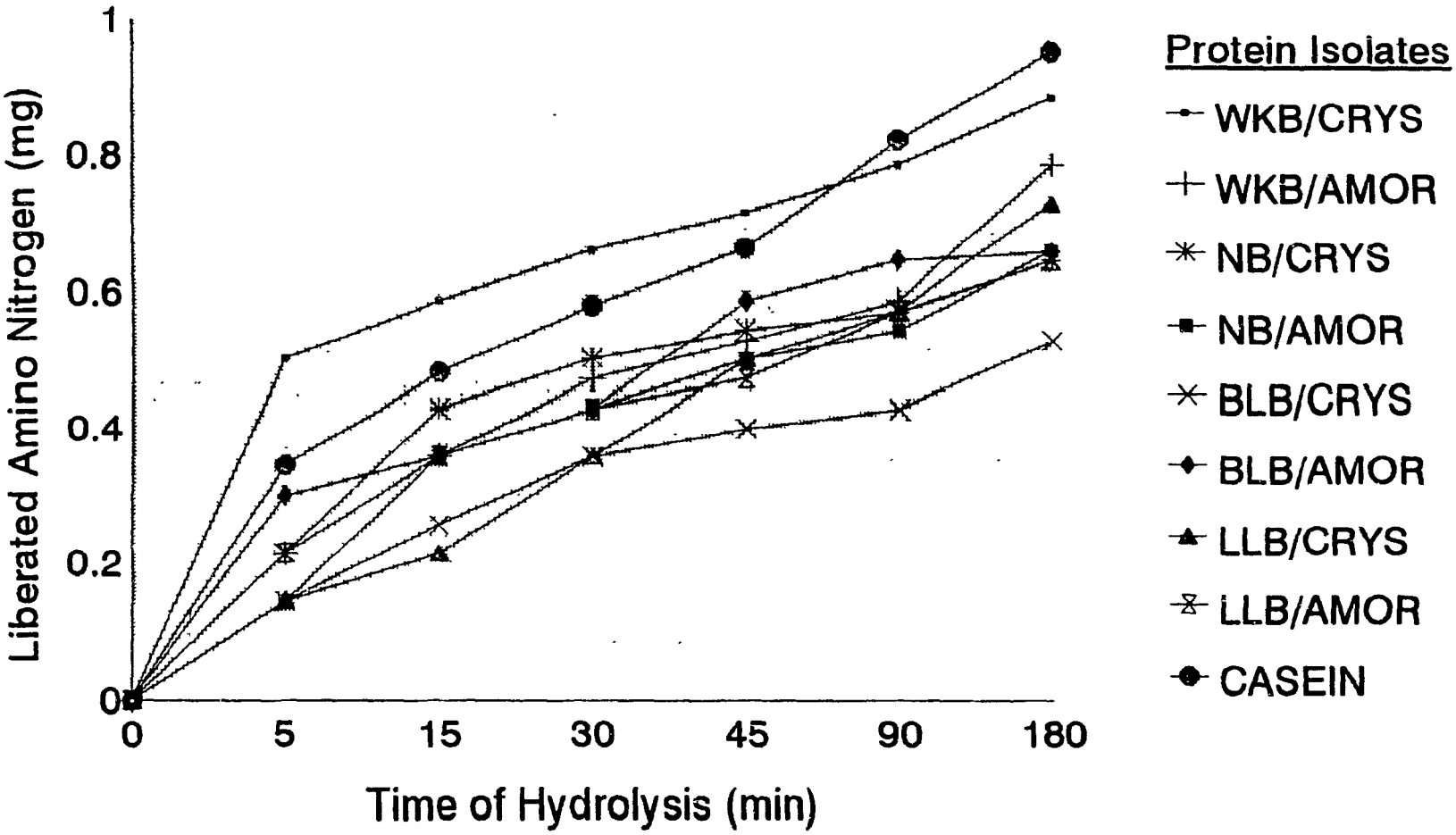

Figure IV.3
A graph of the amount of amino nitrogen (mg/g protein) liberated with time (min), by tryptic hydrolysis of the protein isolates and casein, as determined by Formol titration

IV.B.2. Nitrogen Solubility Index

The nitrogen solubility index (NSI) reflects the solubility properties of the hydrolytic products of the bean protein isolates, and can be used to predict their probable functional properties of the protein hydrolysates (Nakai and Li-Chan, 1988). Figure IV.6 shows the effect of hydrolysis on the nitrogen solubility index (NSI) for all the protein isolates and casein while figures IV.4 and IV.5 show the effect of tryptic hydrolysis on the NSI for the crystalline and the amorphous protein isolates respectively. The results show that the hydrolysates of the crystalline protein isolates of *Phaseolus* beans have much higher solubility properties than the hydrolysates of their amorphous counterparts. The least squares means (LSMEANS) difference (See Appendix, Tables B.1 to B.5) of the NSI between the crystalline and amorphous protein isolates were 11.2% ($P < 0.001$) for NB, 12.96% ($P < 0.001$) for LLB, 16.3% ($P < 0.001$) for BLB, and 29.4% ($P < 0.001$) for WKB. The higher NSI values of the crystalline protein isolates suggests that the constituent protein molecules of the crystalline isolates are more flexible than those of the amorphous protein isolates (Kato et al., 1985; Lee et al., 1992). The results also show that the solubility properties of the hydrolytic products of the crystalline isolates compared with those of the amorphous isolates. This could be due to a higher proportion of albumins (water soluble proteins) in the crystalline protein isolates than the amorphous protein isolates.

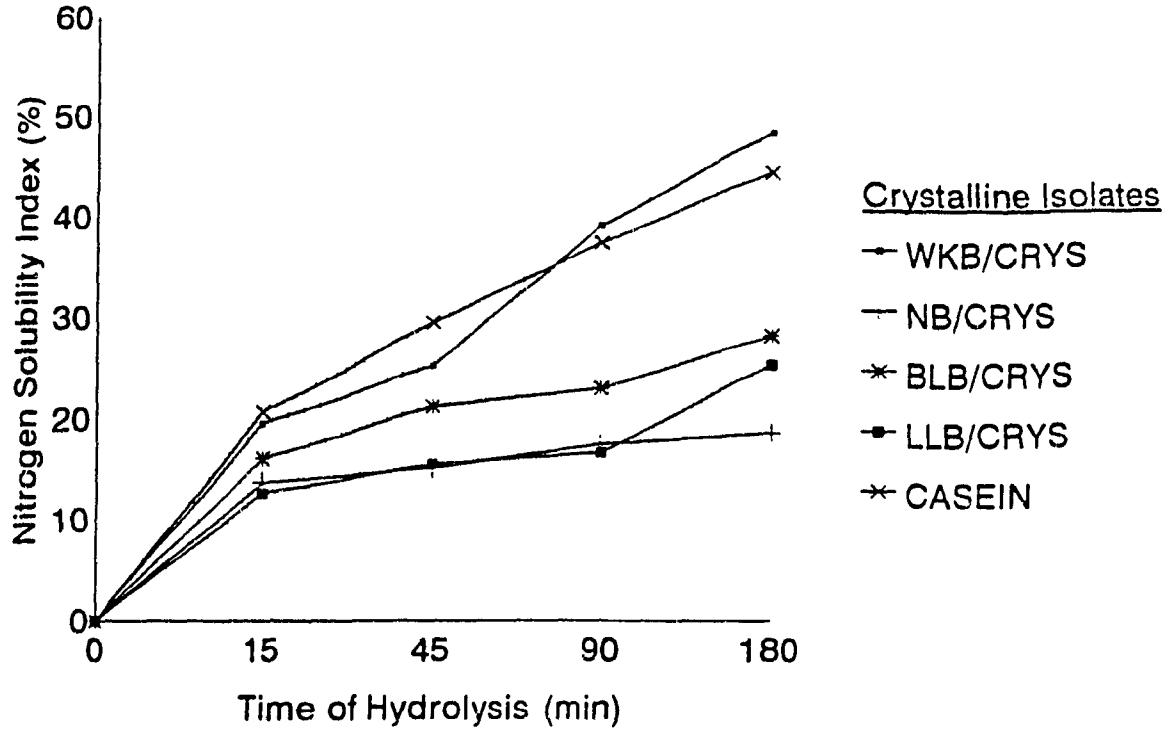

Figure IV.4
A graph of the percentage of protein nitrogen solubilized in 10% TCA by tryptic hydrolysis of the crystalline isolates and casein

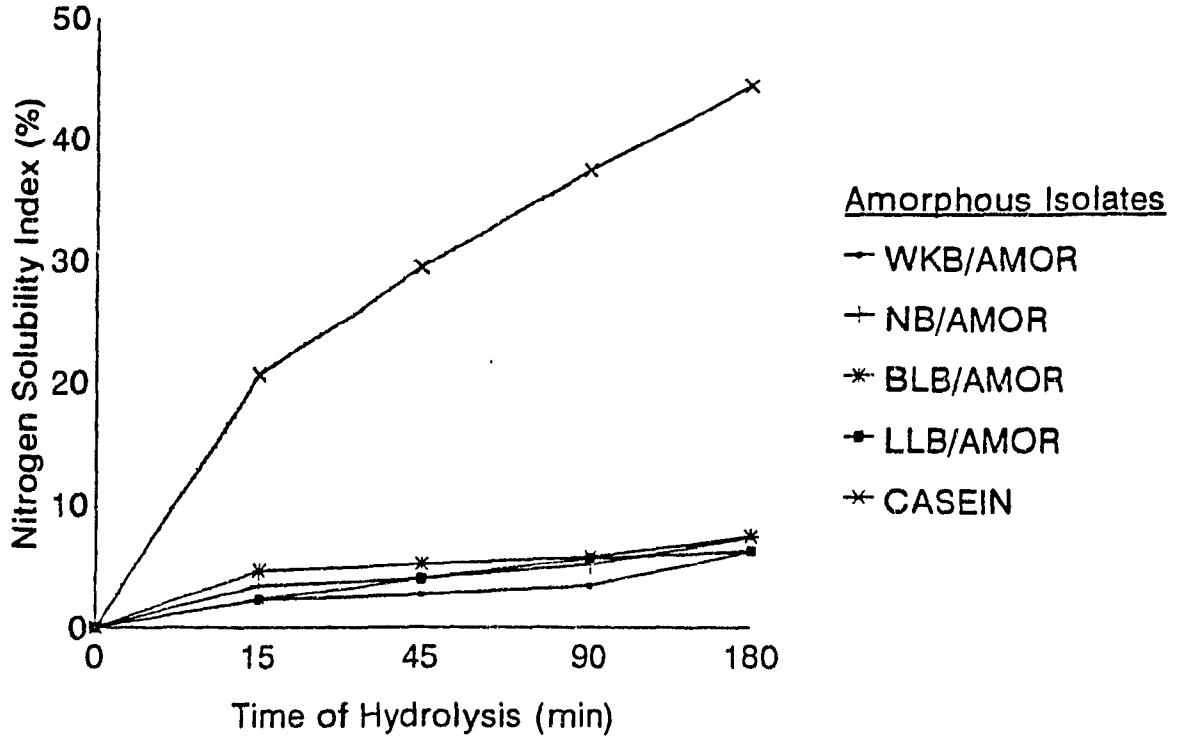

Figure IV.5
A graph of the percentage of protein nitrogen solubilized in 10% TCA by tryptic hydrolysis of the amorphous isolates and casein.

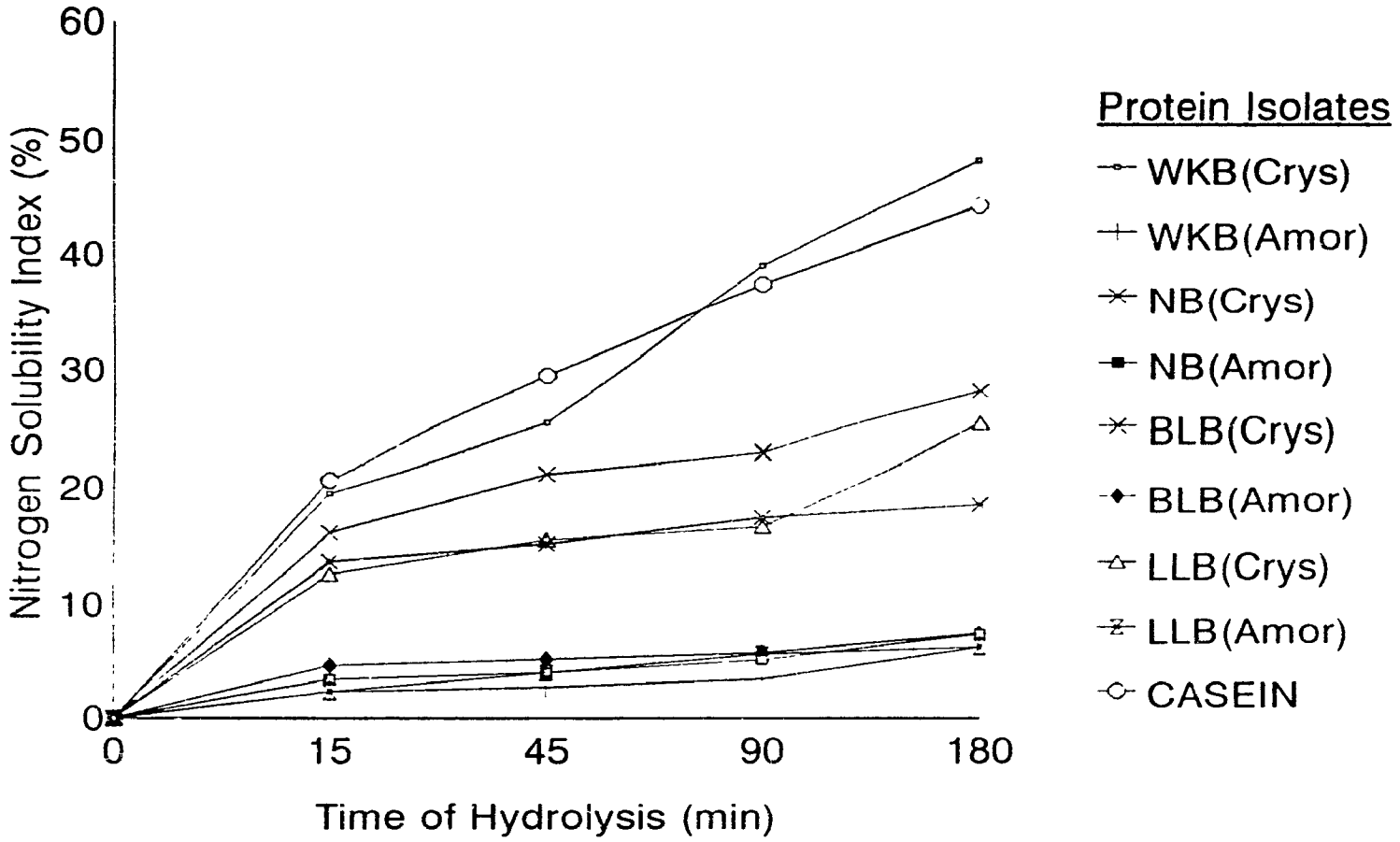

Figure IV 6
The effect of tryptic hydrolysis on the solubility of protein nitrogen in 10% Trichloroacetic acid
Time of hydrolysis at 0 min represents the unhydrolyzed protein

IV.C. Functional Propeties

IV.C.1. Emulsifying capacity (EC)

Figures IV.7 and IV.8 show the effect of hydrolysis on the emulsifying capacity (EC) of the crystalline and amorphous protein isolates respectively, and figure IV.9 shows the effect of hydrolysis on the emulsifying capacity (EC) of all the protein isolates studied and casein. The results show that limited hydrolysis (15 to 30 min hydrolysis) improved the EC of the bean protein isolates, and that extensive hydrolysis (> 45 min hydrolysis) progressively reduced the emulsifying capacity of the proteins. Figure IV.7 show that the emulsifying capacity of the crystalline protein isolates increased to a maximum within 15 min of hydrolysis and figure IV.8 show that the emulsifying capacity of the amorphous isolates increased to a maximum after 30 min of hydrolysis. The results also show that the hydrolysates of the crystalline protein isolates showed higher EC. compared with the hydrolysates of the amorphous protein isolates (Fig. IV.9), and the hydrolysates of casein (Fig IV.7). With the exception of the amorphous protein isolate of WKB which showed an unusually high EC, native casein exhibited higher EC compared with the bean protein isolates (Figs. IV.7 and IV.8).

The improved EC of the bean protein isolates upon limited hydrolysis suggests that the high molecular weight peptide of the protein isolates are more effective in reducing oil/water interfacial tension than the protein isolates. This can be explained by the higher rate of diffusion, and molecular flexibility (Meste et al., 1990) of the high molecular peptide compared with the native proteins. The

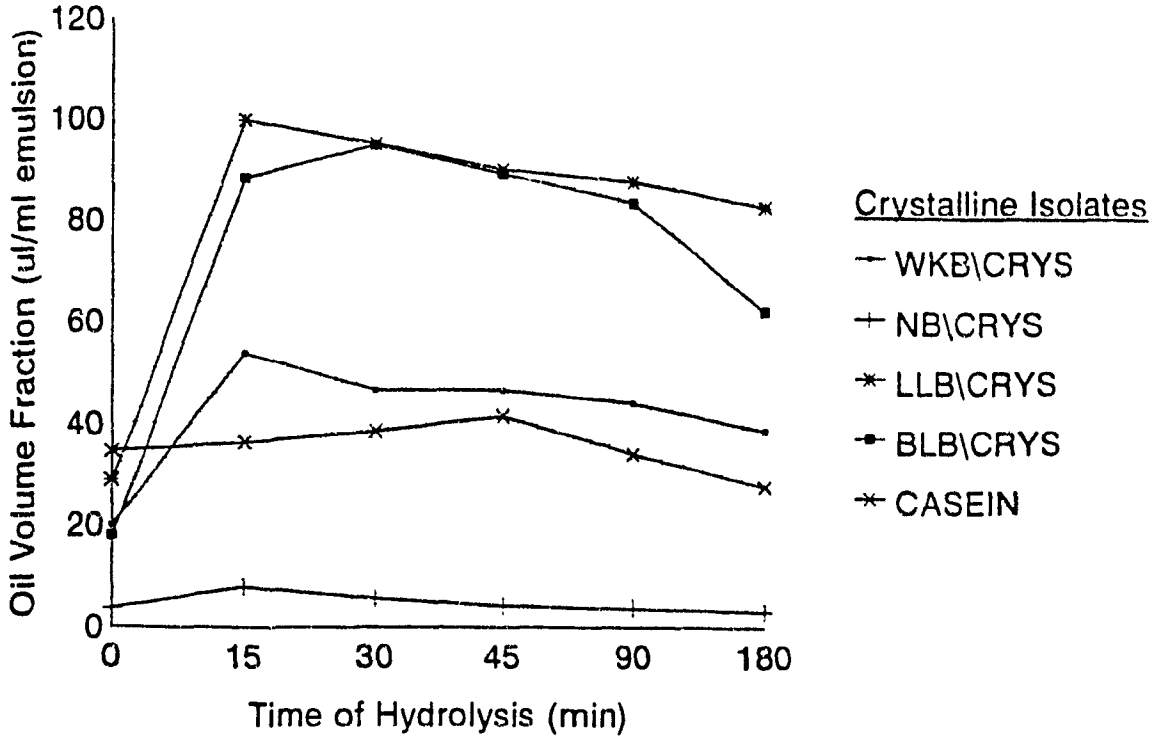

Figure IV.7
Volume of oil (ul/ml emulsion) emulsified by tryptic hydrolysates of the crystalline isolates and casein.

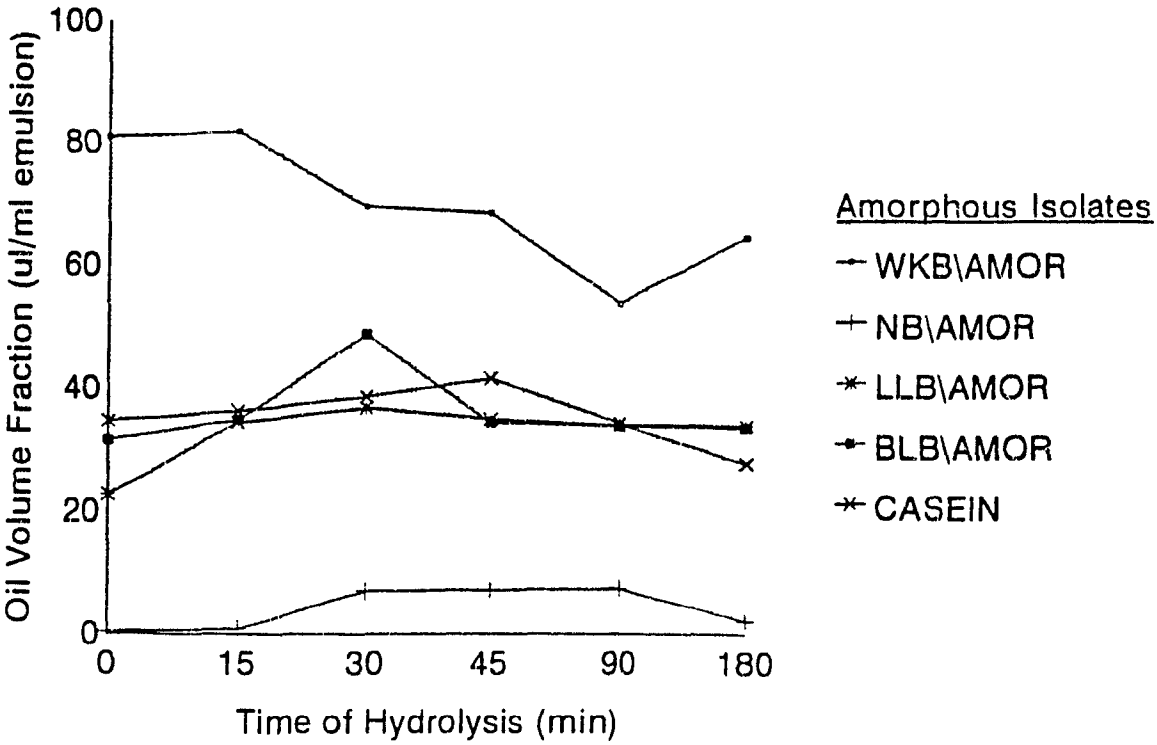

Figure IV.8
Volume of oil (ul/ml emulsified) emulsified by tryptic hydrolysates of the amorphous isolates and casein.

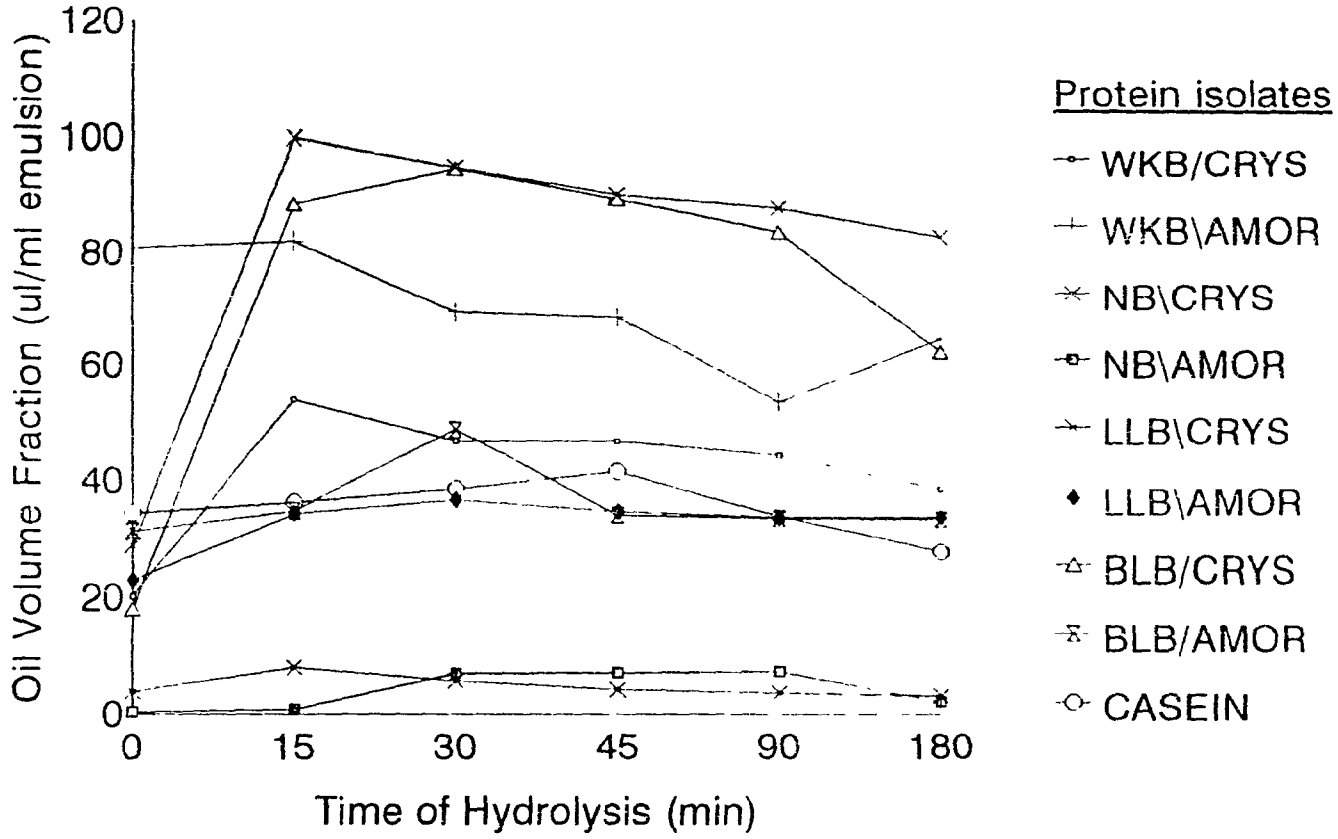

Figure IV.9
The effect of tryptic hydrolysis on the oil volume emulsified by the protein isolates and casein. Hydrolysates at 0 min refers to the unhydrolyzed proteins

improved emulsifying capacity, as a result of limited hydrolysis could also be due to an increase in the net hydrophobicity by the exposure of the interior hydrophobic regions of the native protein, without production of enough hydrophiles (amino and carboxyl groups) to offset the hydrophobicity of the exposed hydrophobic amino acid residues. The progressive decrease in emulsifying capacity with increasing hydrolysis could be due to increasing zeta potential or net hydrophilicity with hydrolysis (Mahmoud et al., 1992). Other workers (Casella and Whitaker, 1990; Turgeon et al., 1992; Haque, 1993) have also shown that small peptide are poor emulsifiers.

The higher EC of the hydrolysates of the crystalline protein isolates compared with the hydrolysates of the amorphous protein isolates could be due to the higher solubility properties of the hydrolytic products of the crystalline protein isolates compared with the hydrolytic products of the amorphous protein isolates (See Section IV.B.2) The higher solubility of the hydrolytic products of the crystalline protein isolates suggests that they will diffuse faster to the oil/water interface during the emulsification process, and protect newly formed oil droplets from re-coalescing (Li-Chang et al , 1984, Lee et al , 1992)

The observation that the crystalline protein isolates attained their maximum EC after only 15 min hydrolysis, while it took up to 30 min hydrolysis for the amorphous protein isolates reached their maximum EC, suggests that the hydrophobic regions of the crystalline proteins isolates are probably oriented more towards the surface of the native proteins, so that a lesser extent of hydrolysis is

required to expose the interior hydrophobic sites necessary for adsorption, and protein-protein interaction at the oil/water interface. This is supported by the higher surface hydrophobicity of the crystalline isolates of *Phaseolus* beans compared with the amorphous protein isolates (Di Lollo, 1990).

The higher EC of native casein (35 μl oil per ml emulsion), compared with the EC of the bean protein isolates (ranging from 2 to 32 μl oil per ml emulsion; with the exception of the amorphous isolates of WKB), can be attributed to the more compact, and rigid native structure of the constituent proteins of the legume protein isolates (Nielsen et al., 1988; Deshpande and Damodaran, 1990, 1991b). Rigid protein molecules adsorb poorly at oil/water interfaces, and are unable to unfold and spread out adequately at interfaces to reduce the interfacial tension (Townsend and Nakai, 1983; Meste et al., 1990).

IV.C.2. Emulsion Stability (ES)

IV.C.2.1. Interfacial area of emulsion

The results of the effect of hydrolysis on the interfacial area coated by the protein isolates is shown in Figure IV.10. The results show that with the exception of the protein isolates (crystalline and amorphous) of NB, tryptic hydrolysis had little effect on the ability of the protein isolates to coat oil/water interfaces. The results also show that there is not much difference in the ability of crystalline and amorphous protein isolates, and their hydrolysates to coat oil/water interfaces (Figure IV.10). Limited hydrolysis (15 to 30 min hydrolysis) however, increased the interfacial area coated by all the protein isolate studied.

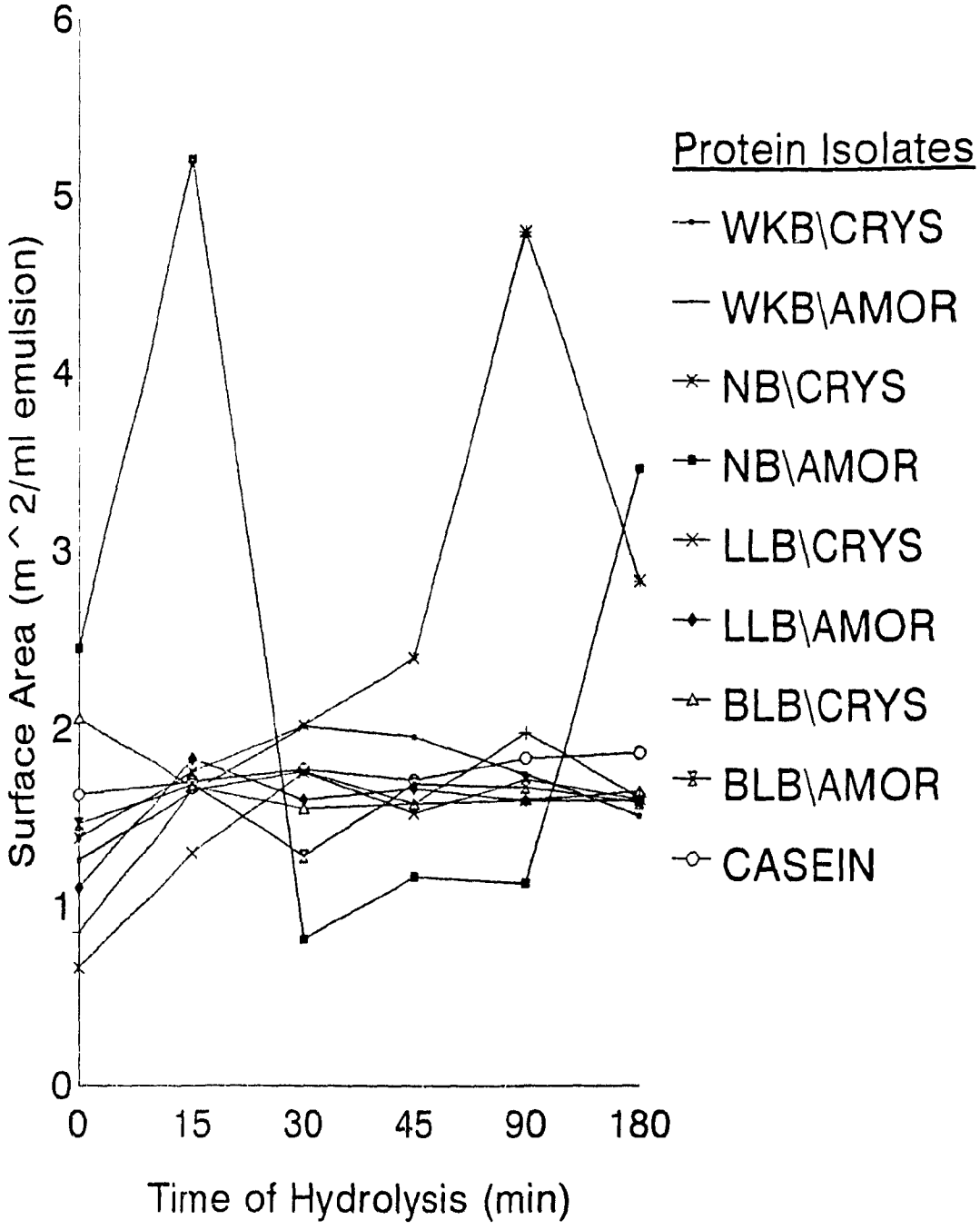

Figure IV.10
Effect of tryptic hydrolysis on the surface area (m ^ 2/ml emulsion) of emulsions formed and stabilized by the protein isolates and casein.

Continued hydrolysis (after 30 mins hydrolysis) did not significantly affect the interfacial area coated by the protein hydrolysates. The capacity of proteins to stabilize emulsions can be related to the interfacial area that can be coated by the protein (Pearce and Kinsella, 1978). Since hydrolysis had little effect on the interfacial area of the emulsions stabilized by the protein isolates, it can be concluded that tryptic hydrolysis did not really affect the ability of the protein isolates to coat the oil/water interfacial area.

IV.C.2.2. Oil Droplet Size

Figure IV.11 shows the results of the effect of hydrolysis on the average droplet size of the emulsions stabilized by the protein isolates, as determined by turbidity measurements for the protein isolates and casein The results show that with the exception of the amorphous protein isolate of WKB, limited tryptic hydrolysis (15 to 30 min hydrolysis) increased the average droplet size of the emulsions stabilized by the protein isolates of *Phaseolus* beans. After an optimum degree of hydrolysis, where the average droplet radius were highest, further hydrolysis progressively decreased the droplet size of the emulsions.

The results also show that the average droplet radius of the emulsions stabilized by the unhydrolyzed crystalline protein isolates were smaller (< 5 μm) compared with those of the unhydrolyzed amorphous isolates (> 5 μm). The effect of tryptic hydrolysis was also more pronounced on the droplet size of the emulsions stabilized by the crystalline protein isolates than those of the emulsions stabilized by the amorphous protein isolates. Limited hydrolysis (15 to 30 min hydrolysis) increased the average droplet radius from 4.8 μm to a maximum of 10 microns (for the emulsion stabilized by the crystalline protein isolate of WKB); from 1.3 μm to a maximum of 23 μm (for the emulsion stabilized by the crystalline protein isolate of LLB); and from 2.6 μm to a maximum of 18.5 μm (for the emulsion stabilized by the crystalline protein isolate of BLB), while the average droplet size of the emulsion stabilized by the amorphous protein isolates remained relatively unchanged with hydrolysis. The smaller droplet size of emulsions stabilized by the crystalline protein isolates compared with those of emulsions

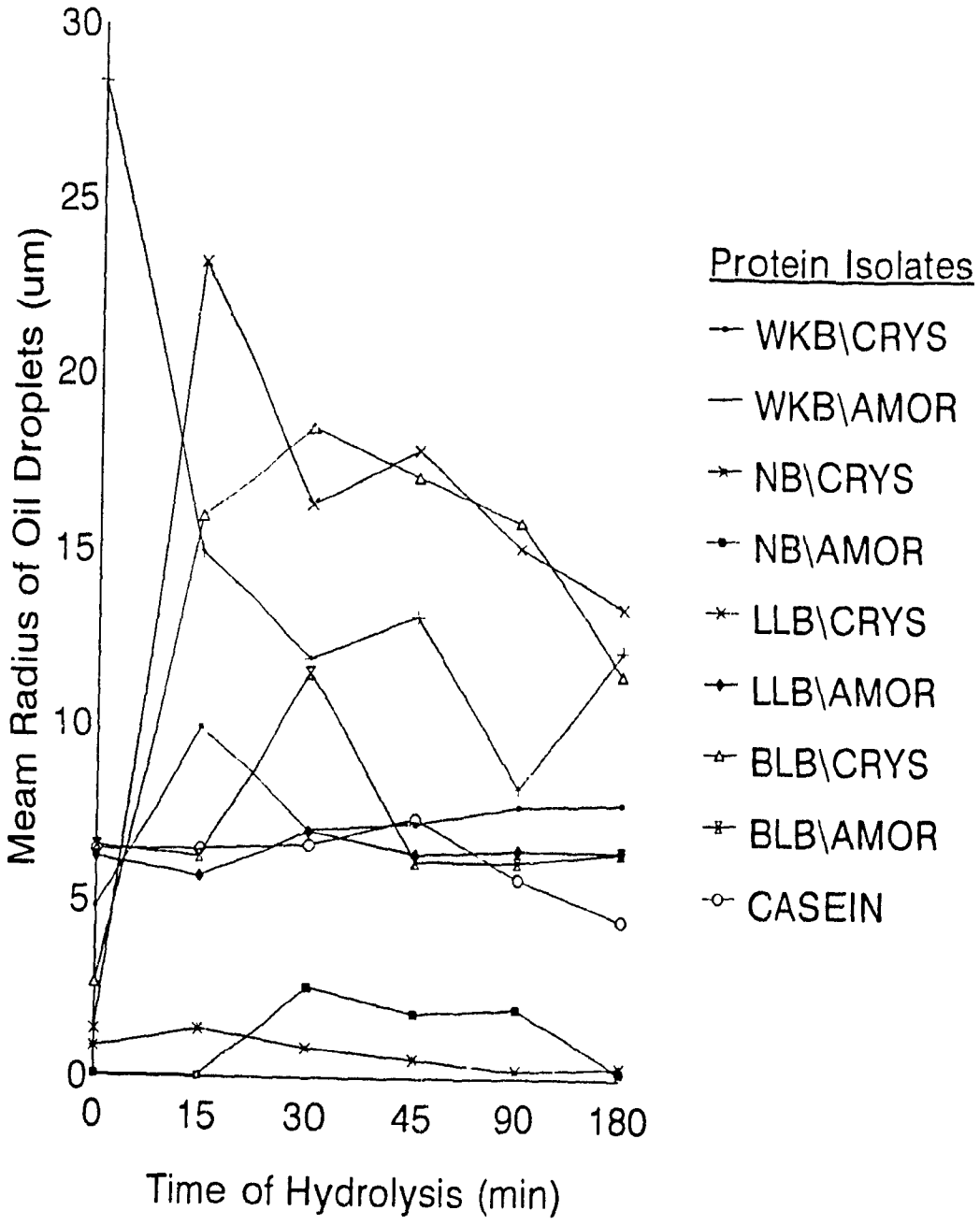

Figure IV.11
Effect of hydrolysis on the radius of the dispersed phase of the emulsions formed and stabilized by the protein isolates and casein.

stabilized by the amorphous protein isolates suggests that the crystalline isolates are more rigid native structure than the amorphous protein isolates.

The increase in emulsion droplet size with limited hydrolysis (< 45 min hydrolysis) suggests that the more flexible polypeptides, with more exposed hydrophobic sites that result from limited protein hydrolysis may have interacted with each other at the interface (through hydrophobic/hydrophobic interaction) to form flexible two dimensional monolayers (Lee et al., 1987) that are able to stretch out and coat large oil droplets. The decrease in droplet size after prolonged hydrolysis (> 45 min hydrolysis) on the other could be due to the production of small peptide which are less hydrophobic, and less capable of interacting effectively with each other at the interface to form strong elastic monolayer.

The protein isolates (crystalline and amorphous) of NB and their corresponding tryptic hydrolysates showed very poorer emulsifying capacity (Figure IV.9), and produced emulsions with very smaller droplet size (< 1 μm) (Figure IV.11) than casein and the other bean protein isolates. This results suggest that, of all the bean protein isolates, the constituent proteins of the protein isolates of navy bean (NB) have very tight and rigid native structures.

The average droplet radius of the emulsions stabilized by proteins is related to the interfacial area coated by the protein, hence the emulsion stability (Pearce and Kinsella, 1978; Lee et al., 1992). While the interfacial area suggests the ability of proteins to stabilize emulsions by coating oil droplets (Pearce and Kinsella, 1978), the droplet size determines the mechanism of emulsion destabilization ie.,

creaming, flocculation, and coalescence (Bergenstahl and Claesson, 1990; Holcomb et al., 1990) Small droplets tend to destabilize emulsions through flocculation and creaming, and large droplets tend to destabilize emulsions through coalescence and creaming. Flocculation and creaming are usually reversible as long as there is no serum separation, however, coalescence is associated with release of free oil from oil/water emulsion leading to emulsion breakdown (Dickinson and Stainsby, 1987). Droplet size distribution of the dispersed phase of emulsions can therefore be used as a predicator of emulsion stability (Holcomb et al., 1990). A decrease in droplet size by a factor of two may decrease coalescence rate by a factor of 10 to 100 (Bergenstahl and Claesson, 1990).

In predicting the stability of the emulsions stabilized by the unhydrolyzed proteins, the emulsions stabilized by the crystalline protein isolates will likely be stable than those stabilized by the amorphous protein isolates. The large droplet sizes of the emulsions stabilized by the hydrolysates of crystalline protein isolates suggests that those emulsions will be less stable than the emulsions stabilized by the hydrolysates of the amorphous isolates. This may not necessarily be so, since the high concentration of the dispersed phase of the emulsions stabilized by the hydrolysates of the crystalline isolates can stabilize those emulsions against creaming (Bergenstahl and Claesson, 1990) It must be noted however that droplet size and density are not the only factors that affect the destabilization kinetic of emulsions (Klemaszewski et al., 1992). Such factors as the interfacial film thickness or viscosity and droplet surface properties such as charge (Bergenstahl

and Claesson, 1990) also affect emulsion stability. From the results, it can also be concluded that hydrolysis decreased the emulsion stabilizing ability of the protein isolates of *Phaseolus* beans.

IV.C.3. Foaming Capacity

Results of the effect of tryptic hydrolysis on the foam expansion capacity of the protein isolates (Table IV.3), shows that hydrolysis improved the foaming capacity of the protein isolates and casein. The amorphous protein isolates of the beans of *P. lunatus* and their corresponding hydrolysates showed higher foaming capacity (foam expansion ranging from 220% to 242% for BLB, and 205% to 225% for LLB) than their crystalline counterparts (foam expansion ranging from 190% to 210% for BLB, and 170% to 190% for LLB). On the other hand the crystalline isolates of the beans of *P. vulgaris* and their corresponding hydrolysates, particularly that of white kidney beans showed higher foaming capacity than their amorphous counterparts.

The higher foaming capacity of the amorphous protein isolates of the beans of *P. lunatus* compared with their crystalline counterparts can be explained by the more flexible structure of the amorphous isolates. The higher foaming capacity of the crystalline isolates of the beans of *P. vulgaris* compared with their amorphous counterparts on the other hand could be due to a higher total hydrophobicity of the crystalline isolates compared with their amorphous counterparts. Foam expansion capacity depend on the total protein hydrophobicity (Nakai and Powrie, 1981)

The enhanced foam expansion capacity of the proteins upon limited hydrolysis (30 to 45 min hydrolysis) can be attributed to the exposure of interior hydrophobic regions of the native proteins, thereby increasing the effective total hydrophobicity (hydrophobic regions in contact with protein environment)

Table IV.3. Foam Expansion (%) of the tryptic hydrolysates of the protein isolates The hydrolysates at 0 min (hydrolysis) represent the unhydrolyzed proteins

Time	NB(C)	NB(A)	WKB(C)	WKB(A)	LLB(C)	LLB(A)	BLB(C)	BLB(A)	Casein
0 min	175	195	215	190	170	205	190	220	180
15 min	190	205	220	206	170	207 5	200	222 5	192
30 min	200	210	230	217 5	190	215	210	242 5	210
45 min	210	215	230	217 5	185	222 5	210	235	220
90 min	210	215	230	215	185	225	210	230	220
180 min	215	215	230	215	175	225	210	230	225 5

Note The values are averages of duplicate runs

(Mahmoud et al., (1992). Limited hydrolysis also breaks down the tight folded native structure of proteins, allowing them to unfold and stretch out more efficiently at interfaces and form protein films to entrap air bubbles during foam formation

After 45 min, continued hydrolysis had little effect on the in foaming capacity of the proteins Extensive hydrolysis of proteins generally cause an increase in the zeta potential or the net charge frequency of the protein solution, and an increase in the hydrophilicity of the protein degradation products (Mahmoud et al., 1992), thus increasing the affinity of the hydrolytic products (peptide) for the bulk of solution rather than the interface.

IV.C.4. Serum Drainage

Tables IV.4 to IV.11 show the results of serum drainage profiles of the foams stabilized by the protein isolates and their hydrolysates for the first 30 min after foam formation, and Table IV.12 shows the results of serum drainage profiles of the foams stabilized by casein and its corresponding hydrolysates for the first 30 min after foam formation. The results show that, with the exception of the protein isolates of NB, foams stabilized by the unhydrolyzed proteins showed the least serum drainage over the 30 min time period The higher serum retention ability of foams stabilized by unhydrolyzed proteins compared with their hydrolysates may be due to the higher viscosity of the protein solutions It has been shown that hydrolysis causes a decrease in the viscosity of protein solutions (Turgeon et al., 1992). Proteins have also been reported to form thicker and more

elastic interfacial films with higher surface viscosity that reduces serum flow within the lamellar structure than peptide (Kinsella, 1981, Dickinson and Stainsby, 1982).

Foams stabilized by the protein isolates of beans of *P. vulgaris* (WKB and NB) and their corresponding hydrolysates (tables IV.4, IV 5, IV 6, and IV.7) showed higher serum retention than foams stabilized by protein isolates of *P. lunatus* (BLB and LLB) and their hydrolysates (Tables IV 8, IV 9, IV.10, and IV 11). Serum retained by the foams stabilized by isolates of *P. vulgaris* after 30 min of standing, ranged from 3 ml (for the foam stabilized by the crystalline isolate of WKB after 180 min of hydrolysis) to 6.2 ml (for foam stabilized by the crystalline isolate of NB after 45 min hydrolysis) upon standing for 30 min, while that of the isolates from *P. lunatus* ranges from 2 6 ml (for the amorphous isolate of BLB after 180 min hydrolysis) to 4 ml (for the unhydrolyzed BLB crystalline isolate) after standing for 30 min. The serum drainage profile of casein and its corresponding tryptic hydrolysates was similar to that of protein isolates of *P lunatus*.

Serum drainage promotes foam breakdown in that it causes stiffness of the foam matrix, and bubble growth through air diffusion from smaller bubbles to bigger bubbles (Oswalds ripening), thus collapsing the foam structure.

Table IV.4. The rate of serum drainage from foams stabilized by tryptic hydrolysates of the crystalline protein isolates of Navy bean

Hydrolysis Time (min)	Volume (ml) of serum drained from foam with time						
	2 min	5 min	10 min	15 min	20 min	25 min	30 min
0	4 3	9 2	12	13 8	15	15 2	15 6
15	5 3	9 6	12 6	14 1	14 9	15 4	15 7
30	5 2	10 3	13 2	14 5	15 1	15 7	15 9
45	4 9	8 3	11 1	12 3	13 2	13 7	13 8
90	5 3	10	13	14 6	15 8	16	16 2
180	5 4	10	13 5	15	15 7	16	16 4

Note The values are averages of duplicate runs

Table IV.5. The rate of serum drainage from foams stabilized by tryptic hydrolysates of the amorphous protein isolates of Navy bean

Hydrolysis Time (min)	Volume (ml) of serum drained from foams with time						
	2 min	5 min	10 min	15 min	20 min	25 min	30 min
0	4 9	9 5	13 2	15 2	16 1	16 5	16 8
15	5	9 7	12 2	13 5	13 8	14	14 5
30	5 2	9 3	12	13 3	14	14 3	14 5
45	6	10)	13	14	14 4	14 8	15 1
90	6	10	12 8	14 2	15	15 3	15 7
180	6 6	10 3	12 9	14 3	15	15 4	15 7

Note The values are averages of duplicate runs

Table IV.6. The rate of serum drainage from foams stabilized by tryptic hydrolysates of the crystalline protein isolates of White kidney bean

Hydrolysis Time (min)	Volume (ml) of serum drained from foams with time						
	2 min	5 min	10 min	15 min	20 min	25 min	30 min
0	5 0	9 2	12 9	15 0	16 0	16 5	16 8
15	5 0	9 8	13 0	14 7	15 5	16 0	16 1
30	5 3	9 9	13 2	14 8	15 8	16 1	16 4
45	5 6	10 0	13 3	13 8	15 9	16 2	16 5
90	6 0	10 0	13 4	15 3	16 3	16 8	16 9
180	6 3	10 2	13 8	15 6	16 3	16 9	17 0

Note The values are averages of duplicate runs

Table IV.7. The rate of serum drainage from foams stabilized by tryptic hydrolysates of the amorphous protein isolates of White kidney bean

Hydrolysis Time (min)	Volume (ml) of serum drained from foams with time						
	2 min	5 min	10 min	15 min	20 min	25 min	30 min
0	4 2	8 7	11 4	12 2	12 6	12 7	12 8
15	5 0	8 8	11 8	12 7	13 3	13 4	13 5
30	5 5	9 0	12 0	13 1	13 6	13 8	13 9
45	5 8	9 2	12 5	13 3	14 0	14 3	14 6
90	6 0	10 0	12 8	14 2	15 0	15 3	15 7
180	6 6	10 3	13 0	14 3	15 2	15 4	15 9

Note The values are averages of duplicate runs

Table IV.8. The rate of serum drainage from foams stabilized by tryptic hydrolysates of the crystalline protein isolates of Large lima bean

Hydrolysis Time (min)	Volume (ml) of serum drained from foams with time						
	2 min	5 min	10 min	15 min	20 min	25 min	30 min
0	5 9	10 1	13 0	14 8	15 8	16 1	16 5
15	5 8	10 0	12 8	14 2	14 9	15 5	15 8
30	6 0	10 4	13 3	15 1	15 6	16 4	16 8
45	6 1	10 4	13 5	15 0	15 7	16 2	16 6
90	6 1	10 4	13 5	15 2	16 0	16 2	16 7
180	6 2	10 5	13 5	15 1	16 0	16 2	16 0

Note The values are averages of duplicate runs

Table IV.9. The rate of serum drainage from foams stabilized by tryptic hydrolysates of the amorphous protein isolates of Large lima bean

Hydrolysis Time (min)	Volume (ml) of serum drained from foams with time						
	2 min	5 min	10 min	15 min	20 min	25 min	30 min
0	5 3	10 2	14 0	15 8	16 2	16 5	16 8
15	9 8	12 5	13 0	14 8	15 0	15 2	15 5
30	9 4	12 3	14 2	15 1	15 6	15 8	16 0
45	9 5	12 8	14 7	15 5	15 9	16 2	16 4
90	9 7	13 0	14 9	15 8	16 1	16 3	16 6
180	9 8	13 2	15 0	15 8	16 2	16 4	16 6

Note The values are averages of duplicate runs

Table IV.10. The rate of serum drainage from foams stabilized by tryptic hydrolysates of the crystalline protein isolates of Baby lima bean

Hydrolysis Time (min)	Volume (ml) of serum drained from foams with time						
	2 min	5 min	10 min	15 min	20 min	25 min	30 min
0	3 0	10 4	13 0	14 4	15 2	15 8	16
15	6 0	10 5	13 0	14 5	15 5	15 9	16 1
30	5 4	10 0	13 0	14 8	15 7	16 3	16 8
45	5 2	10 1	12 9	14 4	15 3	16 0	16 2
90	5 6	10 3	13 0	14 7	15 4	16 1	16 1
180	5 6	10 4	13 3	15 0	15 6	16 2	16 4

Note The values are averages of duplicate runs

Table IV.11. The rate of serum drainage from foams stabilized by tryptic hydrolysates of the crystalline protein isolates of Baby lima bean

Hydrolysis Time (min)	Volume (ml) of serum drained from foams with time						
	2 min	5 min	10 min	15 min	20 min	25 min	30 min
0	5 2	11 6	14 8	15 4	16 3	16 5	17 1
15	8 9	13 3	15 0	16 7	16 9	17 1	17 2
30	8 9	12 8	15 2	16 1	16 6	16 8	17 0
45	8 4	13 0	15 5	16 6	17 0	17 1	17 2
90	8 2	13 0	15 5	16 8	17 0	17 1	17 1
180	8 3	13 2	15 8	17 1	17 1	17 2	17 4

Note The values are averages of duplicate runs

Table IV.12. The rate of serum drainage from foams stabilized by tryptic hydrolysates of Casein

Hydrolysis Time (min)	Volume (ml) of serum drained from foams with time						
	2 min	5 min	10 min	15 min	20 min	25 min	30 min
0	5 6	10 4	13 2	14 8	15 4	15 9	16 0
15	4 0	8 8	13 3	15 0	16 0	16 2	16 3
30	4 8	9 7	14 5	15 4	16 2	16 5	16 6
45	5 4	10 0	14 8	16 1	16 3	16 7	16 7
90	5 5	10 1	14 9	16 2	16 6	16 7	16 9
180	5 8	10 2	15 2	16 5	16 7	16 8	17 2

Note The values are averages of duplicate runs

IV.C.5. Foam Stability

Table IV.13 show the results of foam stability of the foams formed from the solutions of the protein isolates and their hydrolysates, after standing for 30 min. The results show that foam stability reduced with hydrolysis. In the case of the foams stabilized by the protein isolates of NB, stability increased with hydrolysis to a maximum of 100% stability at 30 min of tryptic hydrolysis. Further hydrolysis however caused a decrease in foam stability to 86.9% at 180 min of hydrolysis. The decrease in foam stability with hydrolysis of the protein hydrolyzed foams can be explained by the weakened cohesive interaction of the hydrolytic products at the interface compared with protein-protein interaction at the interface. Serum draining through the lamellar structure of the foam tower under gravity may also dissolve with it protein or peptide molecules that stabilize the foam structure at the interface, thus collapsing the foam structure. The increasing solubility of the hydrolytic products with hydrolysis suggests that, serum drainage induced foam destabilization will increase with hydrolysis.

The results also show that the protein isolates of *Phaseolus* beans and their hydrolysates produced foams with higher stability than casein and its hydrolysates. This suggest that the protein isolates probably have higher total hydrophobicity than casein. Serum drainage induced foam destabilization could also have been more severe in the foams stabilized by casein and its hydrolysates, due to the higher solubility of casein and its hydrolysates compared with the bean protein isolates and their hydrolysates, so that, the adsorbed proteins and peptides at the

Table IV.13. Foam Stability of Hydrolysates After 30 min of Serum Drainage (%)

Time	NB(C)	NB(A)	WKB(C)	WKB(A)	LLB(C)	LLB(A)	BLB(C)	BLB(A)	Casein
0 min	86 7	94 7	100	100	92 8	95 2	100	95 8	62 5
15 min	97 8	90 4	95 8	90 4	86 7	93	95	93 8	32 4
30 min	100	100	92 3	95 6	88 9	91 3	95 4	94 7	45 4
45 min	95 4	95 6	88 4	85 1	82 2	81 6	90 9	92 5	62 5
90 min	90 9	91 3	80 7	78 2	70 5	80	86 3	88 4	33 3
180 min	86 9	86 9	76 9	75 2	66 6	72	84 1	80 7	16

Note The values are averages of duplicate runs

air water interface of the foams stabilized by casein and its hydrolysates could be more eazyly dissolved from the interface by the draining serum.

IV.D. RP-HPLC

IV.D.1. Direct RP-HPLC of Hydrolysates

Figures IV.12, IV.13, IV.14, and IV.15 (A, B, C, D) show the reversed phase HPLC peptide maps for the tryptic hydrolysates of the bean protein isolates of WKB, NB, LLB, and BLB respectively A, B, C, and D represent the respective peptide maps for the hydrolysates at 0, 5, 45, and 90 min of tryptic hydrolysis. The chromatograms at time 0 min represents the peptide maps of the unhydrolyzed protein isolates. The upper chromatograms represent peptide maps of the hydrolysates of crystalline protein isolates while the lower chromatograms represent peptide maps of the hydrolysates of amorphous protein isolates.

Comparing the RP-HPLC peptide maps for the hydrolysates of crystalline and amorphous proteins isolated from the same bean variety, it can be seen that the peptide maps show different patterns for the tryptic hydrolysates of crystalline and amorphous protein isolates at all stages of hydrolysis The peptide maps also show that the hydrolysates of crystalline protein isolate were better resolved into their component peptide by RP-HPLC than the hydrolysates of the amorphous protein isolates, with respect to peak symmetry, and peak width The peak heights of the peptide maps of the crystalline protein isolates were also higher than those of the peptide maps of the amorphous isolates. The results also show that the number of peaks in the chromatograms and their intensities increased with time of hydrolysis. With the exception of the crystalline isolate of white kidney bean (WKB.CRYS), all the unhydrolyzed proteins showed little or no peptide peaks.

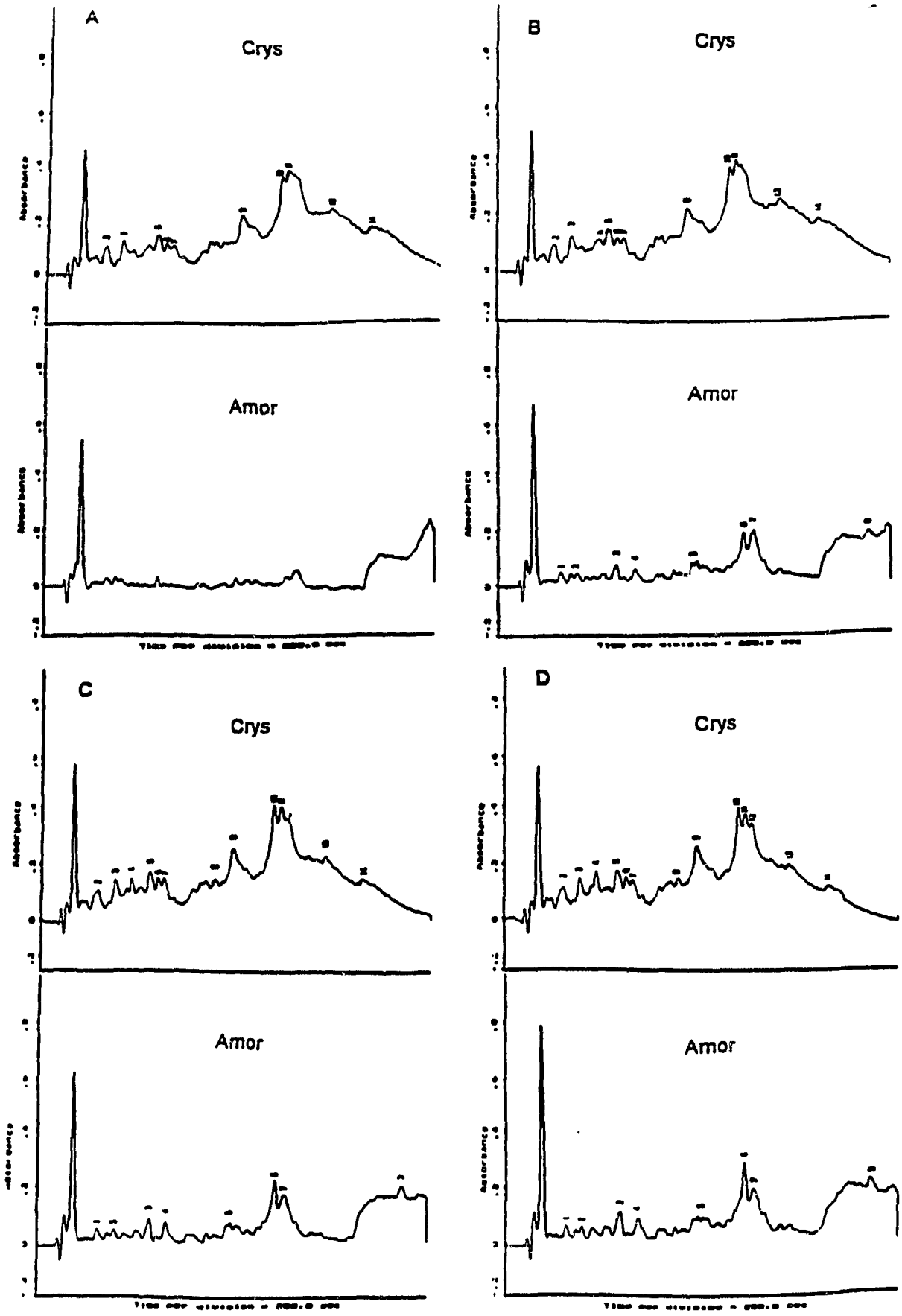

Figure IV.12. Reversed phase-HPLC peptide maps for the protein isolates of white kidney bean (WKB). A, B, C, and D represent the peptide maps for the hydrolysates obtained after 0, 5, 45, and 90 min of tryptic hydrolysis respectively. The hydrolysate at 0 min hydrolysis represent the unhydrolyzed protein solution. The upper chromatograms show the peptide maps of the hydrolysates of the crystalline protein isolates

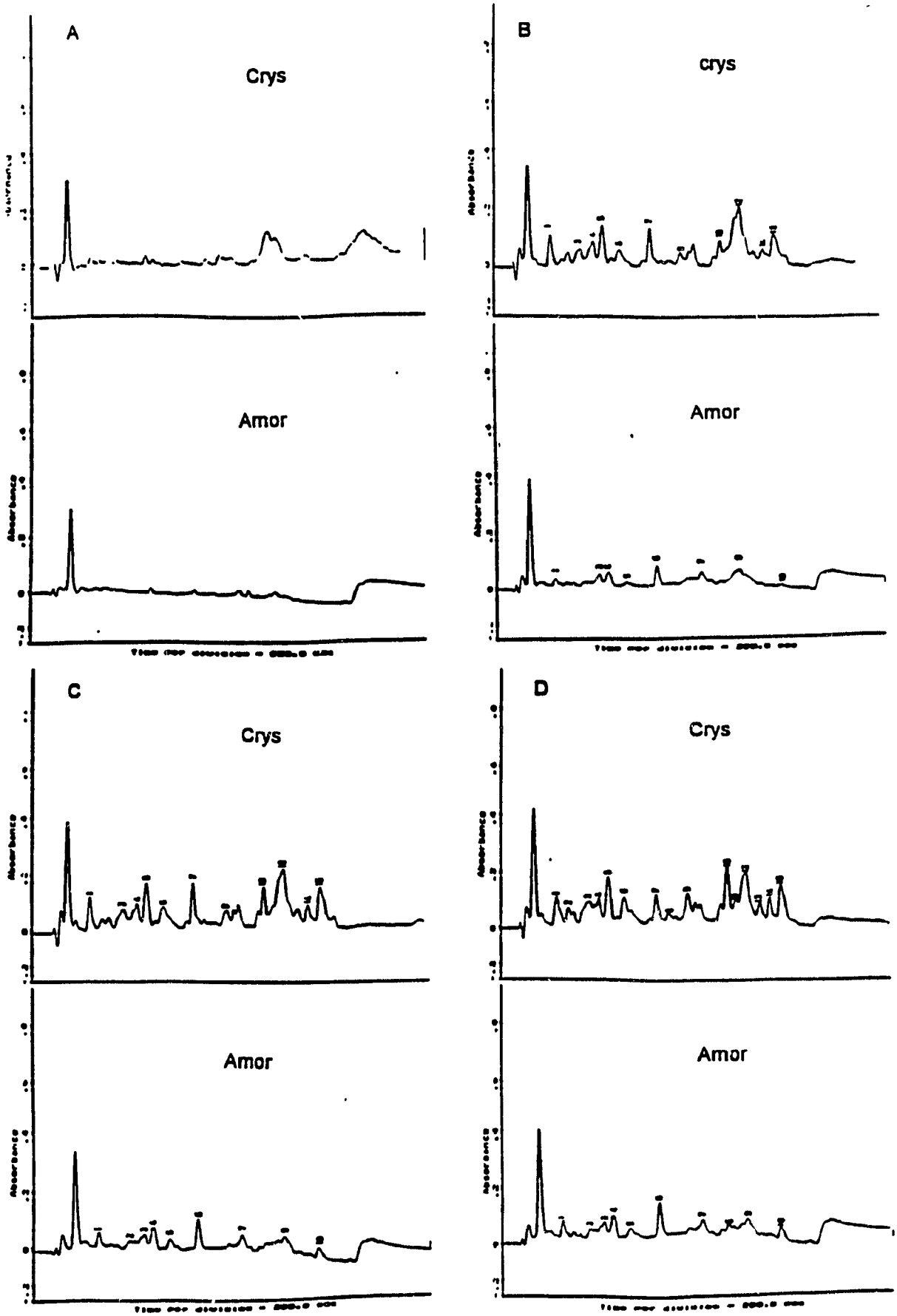

Figure IV.13. Reversed phase-HPLC peptide maps for the protein isolates of navy bean (NB) A, B, C. and D represent the peptide maps for the hydrolysates obtained after 0, 5, 45, and 90 min of tryptic hydrolysis respectively. The hydrolysate at 0 min hydrolysis represent the unhydrolyzed protein solution. The upper chromatograms show the peptide maps of the hydrolysates of the crystalline protein isolates

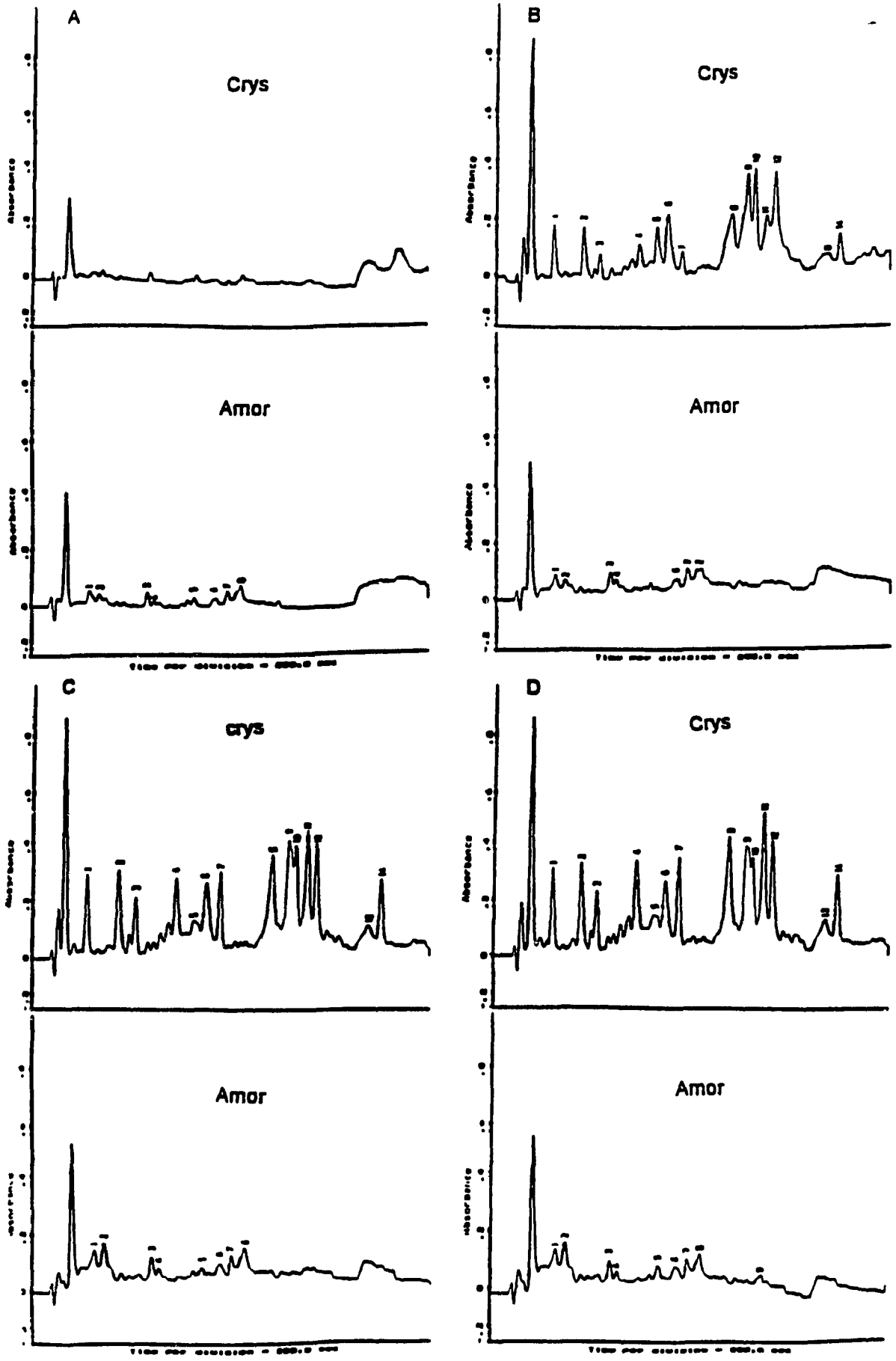

Figure IV.14. Reversed phase-HPLC peptide maps for the protein isolates of large lima bean (LLB). A, B, C. and D represent the peptide maps for the hydrolysates obtained after 0, 5, 45, and 90 min of tryptic hydrolysis respectively. The hydrolysate at 0 min hydrolysis represent the unhydrolyzed protein solution. The upper chromatograms show the peptide maps of the hydrolysates of the crystalline protein isolates.

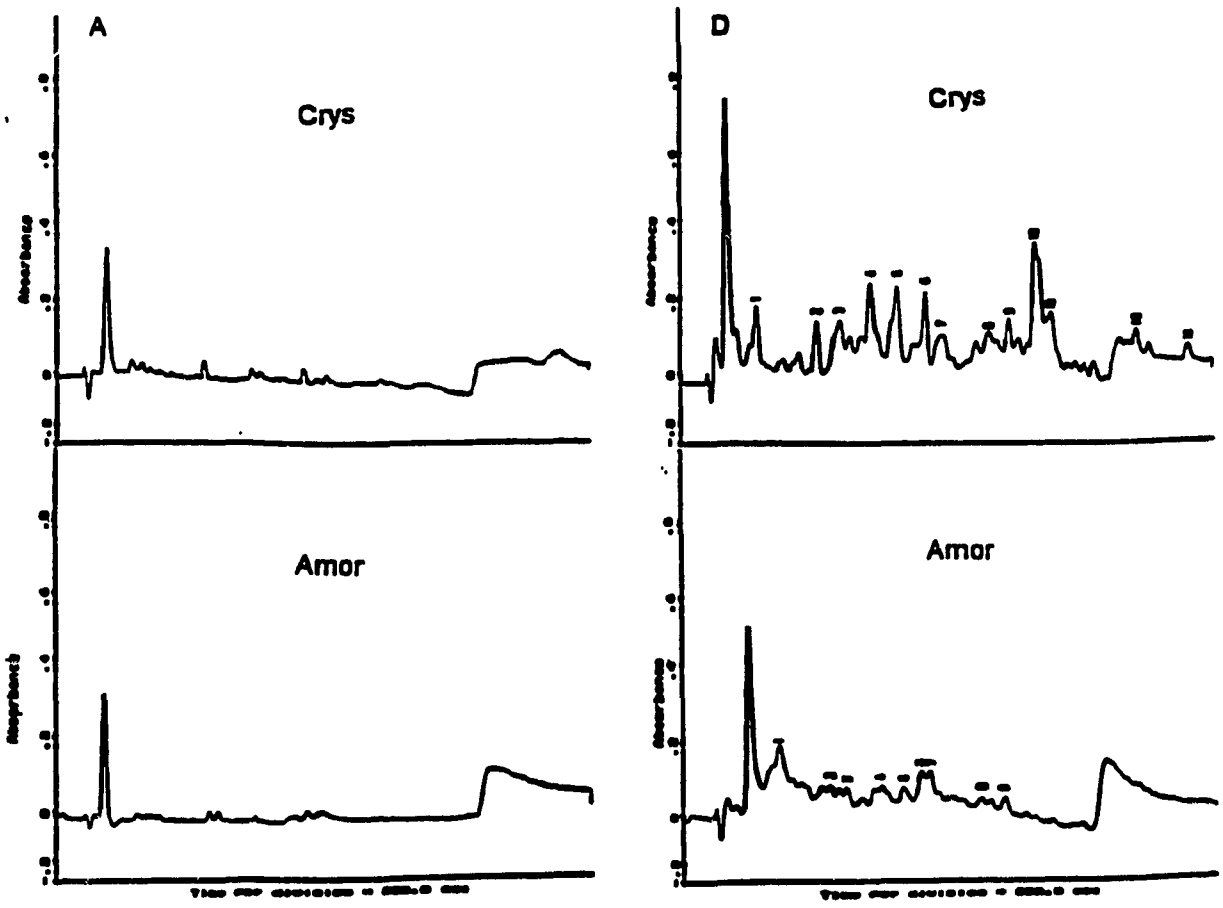

Figure IV.15. Reversed phase-HPLC peptide maps for the protein isolates of baby lima bean (BLB). A, and D represent the peptide maps for the hydrolysates obtained after 0, and 90 min of tryptic hydrolysis respectively. The hydrolysate at 0 min hydrolysis represent the unhydrolyzed protein solution. The upper chromatograms show the peptide maps of the hydrolysates of the crystalline protein isolates.

The difference between the RP-HPLC peptide map patterns of the tryptic hydrolysates of crystalline and amorphous protein isolates from the same bean variety, suggests that the peptide profiles of the crystalline and amorphous protein isolates are different, and that the protein composition of the crystalline and amorphous isolates are also different. The remarkable difference between the peptide map of hydrolysates of crystalline and amorphous bean protein isolates also suggests that peptide mapping can be used to identify proteins with different microstructures. It is possible that the difference between the peptide map pattern of crystalline and amorphous protein isolates from the same bean, and therefore, the protein composition between crystalline protein isolates (citric acid extract) and their amorphous counterparts could be due to the exclusion of certain seed proteins (ie.,proteins with pI in the region of 3.5 to 5) from the citric acid extract, since the pH of citric acid solutions used to isolate the crystalline proteins were in the region of 3.5 to 5.5. The citric acid solutions solubilized up to only 56% of the total seed protein, while dilute NaOH used to isolate the amorphous proteins solubilized over 86% of the total seed protein (see Section IV.A.1).

The better RP-HPLC resolution of the hydrolysates of the crystalline protein isolates compared with that of the hydrolysates of the amorphous protein isolates may be due to the higher purity of the crystalline (see Table IV.2), and the higher solubility properties of the hydrolytic products of the crystalline isolates (See Figure IV.4). The higher solubility properties of the peptide of the crystalline isolates may cause them to attain partition equilibrium (between the stationary phase and the

mobile phase during chromatographic separation) more rapidly in the theoretical plates along the chromatographic bed (column) during the separation process (Ravindranath, 1990) and improve their resolution.

The poor chromatographic resolution of the hydrolytic products of the amorphous isolates compared with those of the crystalline isolates, can also be explained in terms of the resistance of the hydrolytic products of the crystalline isolates to mass transfer in the mobile phase due to poor solubility. Resistance of solute molecules to mass transfer in the mobile phase results in some of the solute molecules in the bulk of the mobile phase moving ahead of those which are near or are at the mobile phase-stationary phase interface, thus causing band broadening and tailing (Ravindranath, 1990). The higher intensities of the peaks in the peptide maps of the hydrolysates of crystalline isolates compared with those of the amorphous isolates could be due to a higher concentration of peptide in the hydrolysates of the crystalline isolates as a result of their higher solubility properties.

Figure IV.16 A, B, C and D show the peptide maps of the tryptic hydrolysates (after 180 min of hydrolysis) of the crystalline protein isolates of WKB, NB, LLB and BLB respectively, and figure IV.17 A, B, C and D show the peptide maps of the tryptic hydrolysates (after 180 min hydrolysis) of the amorphous protein isolates of WKB, NB, LLB and BLB respectively. The results show that among the hydrolysates of the crystalline isolates, those isolated from *P. lunatus* (LLB, and BLB) generated better resolved peptide maps and more intense peaks

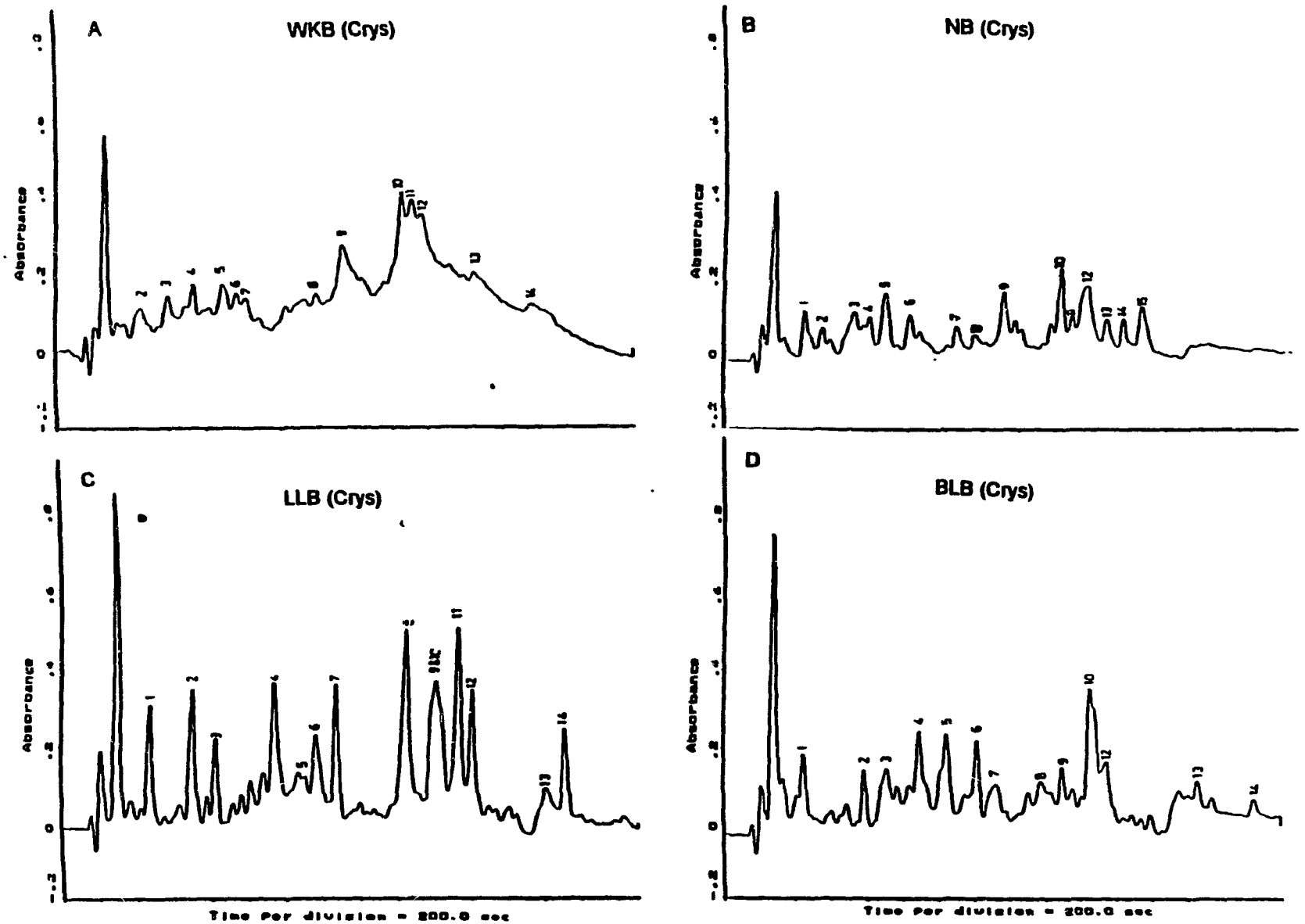

Figure IV.16. Reversed phase-HPLC peptide maps for the tryptic hydrolysates of the crystalline protein isolates obtained after 180 min hydrolysis. A, B, C, and D show the peptide maps of the hydrolysates of the crystalline isolate from Wh NB, LLB, and BLB respectively

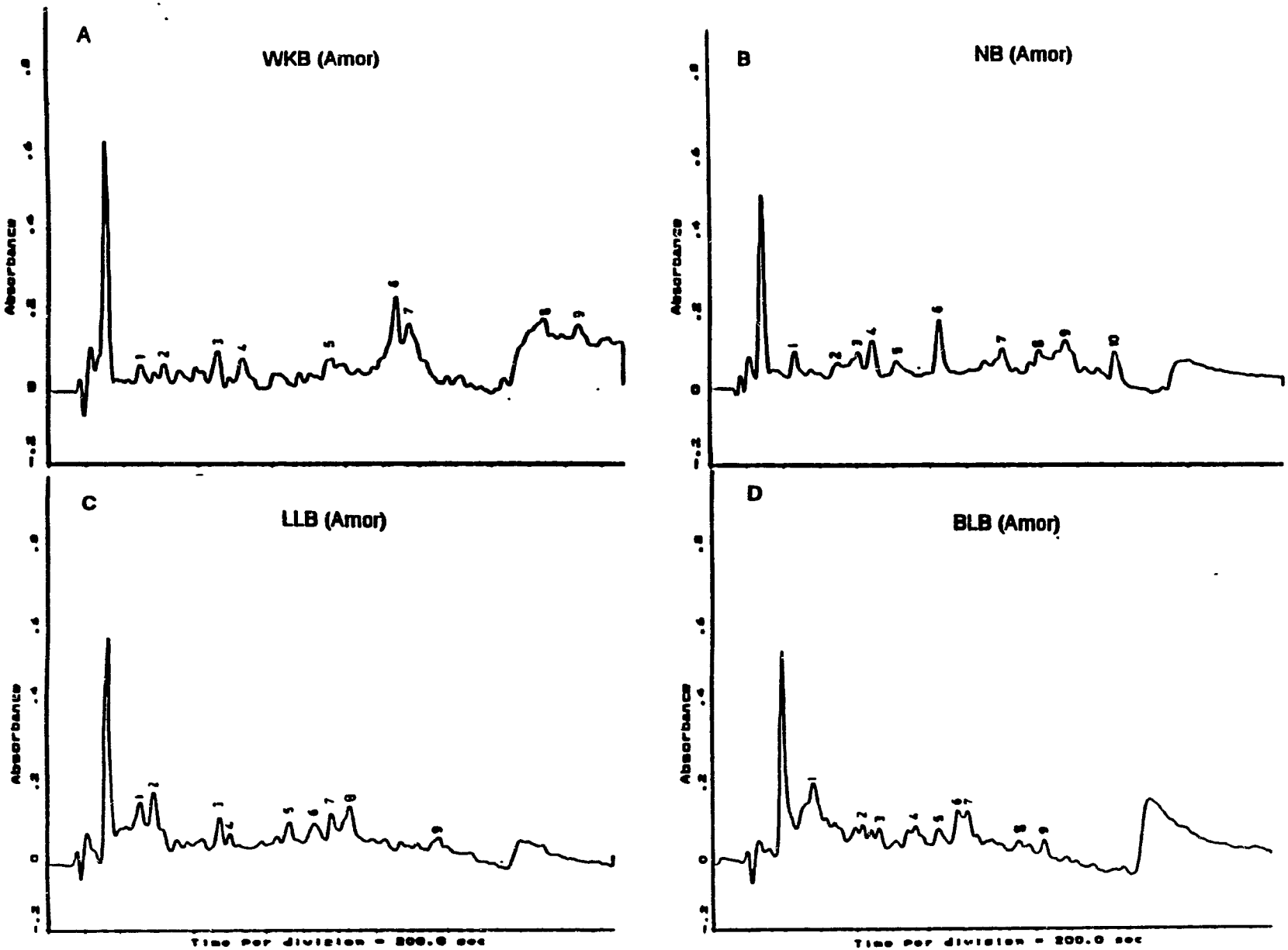

Figure IV.17. Reversed phase-HPLC peptide maps for the tryptic hydrolysates of the amorphous protein isolates obtained after 180 min hydrolysis. A, B, C, and D show the peptide maps of the hydrolysates of the amorphous isolate from WKB, NB, LLB, and BLB respectively

compared with those isolated from *P. vulgaris* (WKB and NB). In the case of the hydrolysates of the amorphous isolates of *Phaseolus* beans, the chromatographic resolution of all the hydrolysates were relatively poor, but those of *P. vulgaris* showed slightly better resolution. In all cases (both crystalline and amorphous) however the difference in the peptide maps is enough to differenciate the protein isolates from each other.

Figures IV.18 show the RP-HPLC peptide maps for the hydrolysates of the crystalline protein isolate of LLB at 0, 5, 30, 45, 90, and 180 min of tryptic hydrolysis respectively. The chromatogram at 0 min represents the RP-HPLC peptide map for the unhydrolyzed protein isolate. The chromatograms clearly show an increase in the number of peaks and peak intensities as hydrolysis proceeds from the unhydrolyzed protein isolate (0 min hydrolysis) through to 180 min of hydrolysis. At a glance, the peak pattern of the RP-HPLC peptide map look similar at all stages of hydrolysis (except at time 0 min). A close inspection of the peptide maps however, reveal that the intensities of some peaks increase relatively with increasing hydrolysis, while the intensities of other peaks either remain unchanged, or decrease relatively with increasing hydrolysis. Figure IV.18 shows that the peak size (height and area) of peaks 4, 7, 8, and 11 increased slowly at the early stages of hydrolysis, but more rapidly at the latter stages of hydrolysis. The peak size (height and area) of other peaks eg., peaks 5, and 10 reached their maximum size at the early stages of hydrolysis (ie., within 15 min of hydrolysis) but decreased progressively as hydrolysis progressed from 15 min to 180 min. Other peaks (eg.,

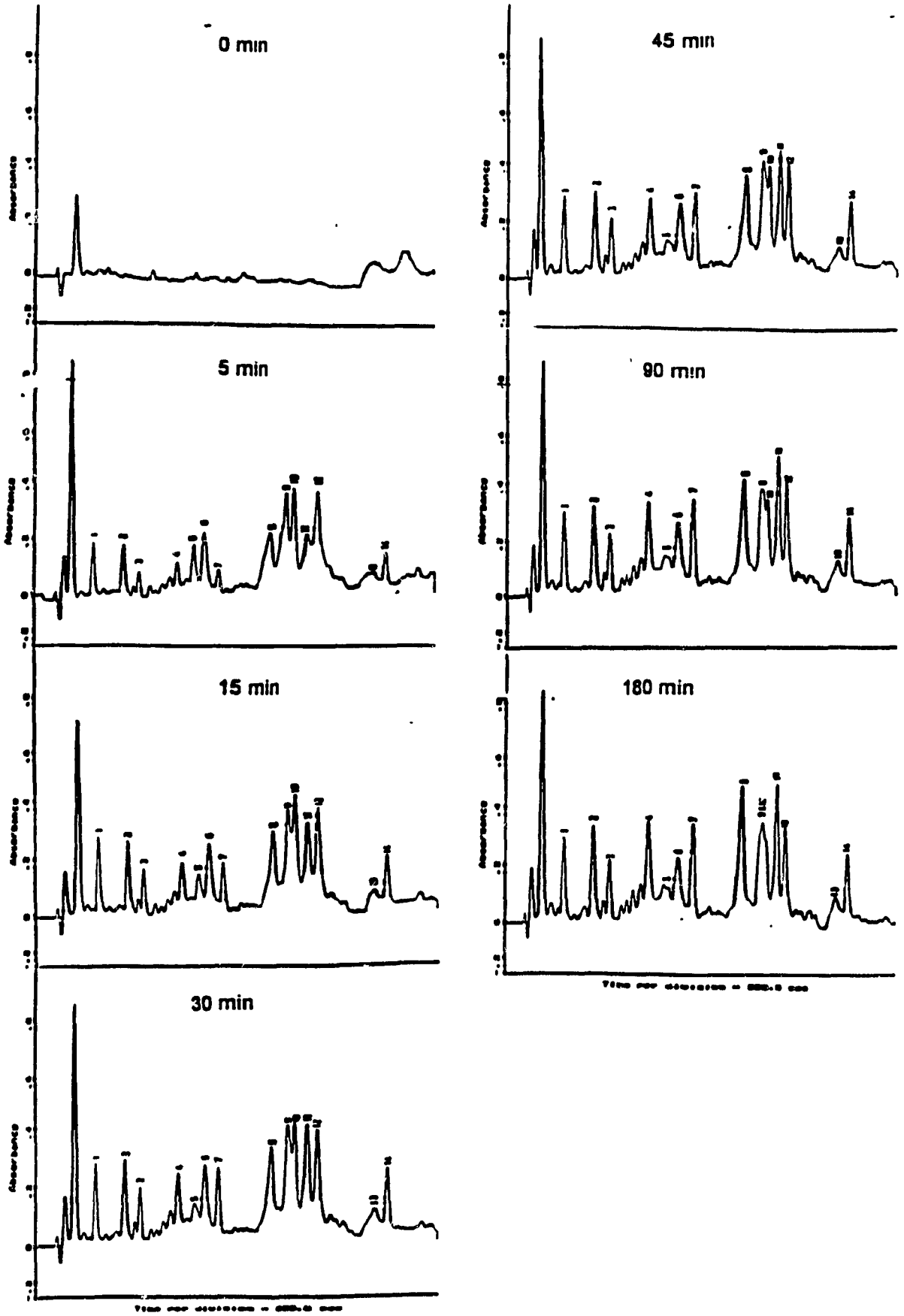

Figure IV.18. Reversed phase-HPLC peptide maps of the tryptic hydrolysates of the crystalline protein isolate from large lima bean, at 0 min hydrolysis (unhydrolyzed protein isolate), 5, 15, 30, 45, 90, and 180 min of hydrolysis.

1, 2, 3, and 14) increased gradually throughout the hydrolytic process.

Peptide whose peak sizes (area and height) reached a maximum at the early stages of hydrolysis and decreased as hydrolysis progressed, suggests that those peptide segments are probably located at or near the surface of the native protein and were therefore easily accessible to the enzyme action at the early stages of hydrolysis. Peptide whose peak size decreased as hydrolysis progressed, suggests that those peptide fragment also acts as a substrate to trypsin after they have been formed. Peptides whose peak size increased slowly at the early stages of hydrolysis, but increased more rapidly towards the latter stages of hydrolysis, suggests that those peptide segments were located in the interior of the native protein, so that they become more accessible to enzyme action as hydrolysis progressed and the interior of the native protein became more exposed to enzyme activity.

The results show that reversed phase HPLC peptide mapping of protein hydrolysates at various time intervals of hydrolysis can be used to characterize the tertiary and quaternary structures of native proteins, and to study the kinetics of *in vitro* hydrolysis of specific sedgements of native proteins.

IV.D.2. Pre-Column Separation of Hydrolysates

Figures 1V.19 A and B show the peptide maps of the hydrolysates of the crystalline isolates of BLB and LLB (*P. lunatus*) after 180 min of tryptic hydrolysis, and the peptide maps of the acidic, basic, and neutral peptide pools obtained after pre-column separation of the corresponding hydrolysates on solid phase cartridges. The results show that the peptide maps of the different peptide pools (acidic, basic and neutral peptide pools) are very different from each other, and from the original protein hydrolysates. In comparing the elution profile of the peptide components of the peptide pools, the results show that the major peptide components of the basic peptide pools were eluted earlier than the major peptide components of the acidic and neutral peptide pools. The results also show that the basic peptide pools contained fewer peptide than the acidic and neutral peptide pools.

The difference in the RP-HPLC peptide maps of the different peptide pools suggests that the peptide profiles of the acidic, basic and neutral peptide pools are different, and that the solid phase extraction cartridges were effective in separating the original hydrolysates into peptide pools with different chemistries. The earlier elution of the basic peptide compared with the acidic and neutral peptide can be explained by a higher solubility of the basic peptide in the mobile phase (Solvent A = 0.1% TFA; Solvent B = 0.09% TFA in acetonitrile: pH 2.2). The basic peptide will be protonated at the amino groups of their basic amino acid residues due to the low pH of the mobile phase, resulting in an increased affinity of the peptide for the mobile phase and decreased affinity for the hydrophobic stationary phase, and

a consequent early elution.

The fewer number of basic peptide in the original hydrolysate (as shown by the peptide map od the basic peptide pools) compared with acidic and neutral peptide (as shown by the peptide maps of the acidic and neutral peptide pools) indicate that the protein isolates contain fewer basic amino acid residues than acidic or neutral amino acid residues. The use of the solid phase extraction cartridges afforded abbreviated peptide pools with less complex peptide composition which could easily resolved into the component peptide by RP-HPLC. The solid phase cartridges also produced concentrated peptide pools thereby eliminating further concentration steps (Spanier and Edwards, 1987; Voirin and Letavernier, 1991).

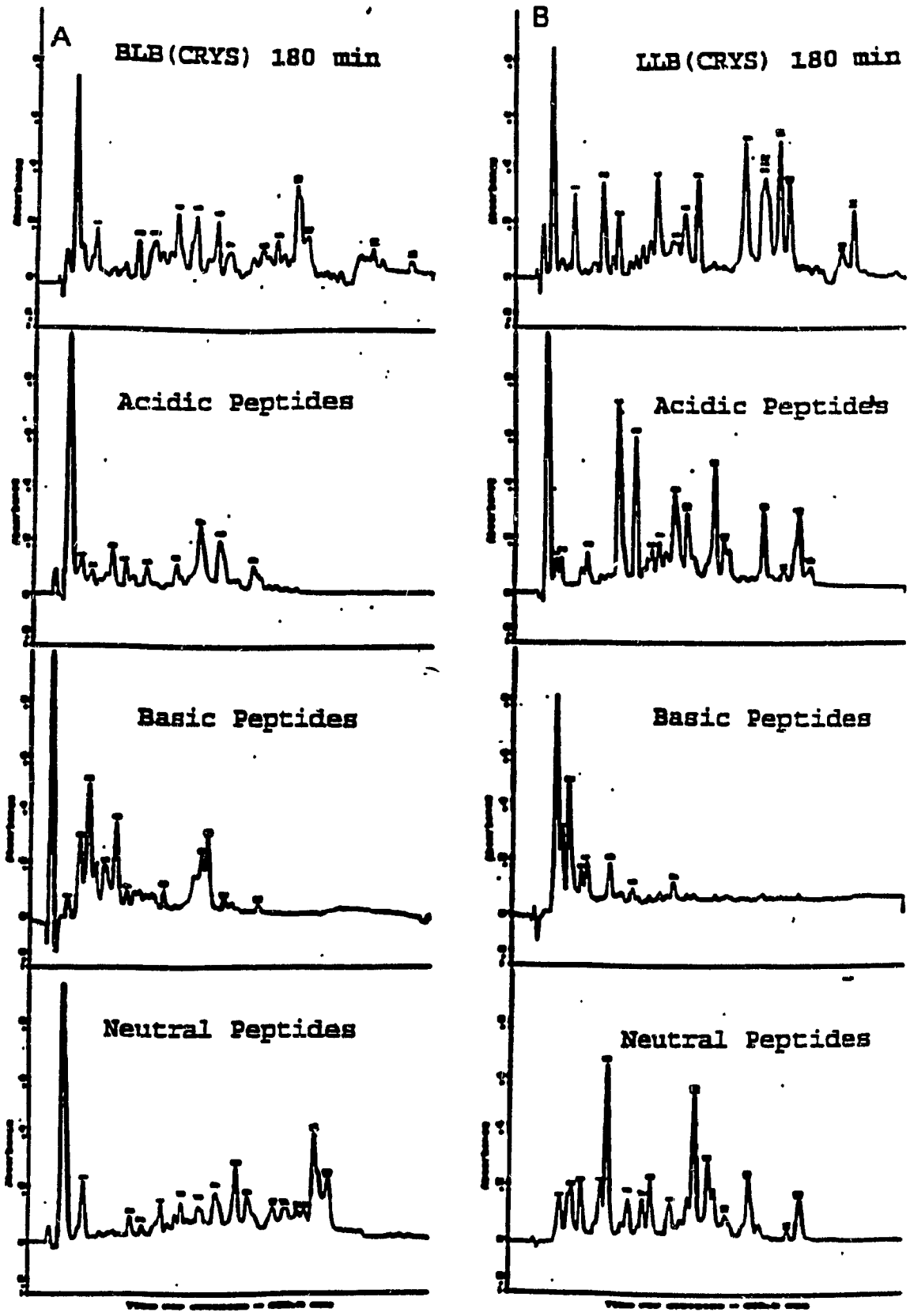

Figure IV.19. Reversed phase-HPLC peptide maps showing the peptide profile of the total hydrolysate obtained after 180 min hydrolysis, and after partitioning the total hydrolysate into acidic, basic, and neutral peptide pools prior to HPLC separation

IV.E.1. ESI/MS Molecular Weight Determination

Figure IV.20A is a typical electrospray ionization (ESI) mass spectrum of a fraction of the RP-HPLC chromatographic separation of the tryptic hydrolysates of the protein isolates. The ESI mass spectrum (Figure IV.20) represents the molecular ion spectrum that defines the peptide components of fraction "7" of the crystalline isolate of navy bean after 180 min of hydrolysis (ie., peak "7" in figure IV.16B). Molecular weight determination of peptide in a mass spectra involves an accurate determination of the charged states of the molecular ion peaks that define the peptide molecules. Equation [IV.1] describes the relationship between the molecular weight of a peptide "P" with a molecular ion peak at m/z "p_1" and a charge "$+z_1$" (z is a positive integer).

$$p_1z_1 = \text{Molecular Weight of peptide "P"} + z_1(M_H) \qquad \text{IV.1}$$

Where M_H is the mass of a proton, assuming the charge carrying species is a proton (H^+). The molecular weight of peptide molecules defined by two or more multiply charged molecular ion peaks, as shown in figure IV.28 can be determined by any two of the molecular ion peaks that define the peptide molecule. If two adjacent multiply charged protonated molecular ions at $m/z_1 = p_1$ and $m/z_2 = p_2$, with charges z_1 and z_2 respectively (where $z_2 > z_1$, and $z_2 - z_1 = 1$), define a peptide molecule "P", then

$$p_1z_1 = \text{Molecular Weight of "P"} + z_1(M_H) \quad \text{IV.2}$$

and

$$p_2z_2 = \text{Molecular Weight of "P"} + z_2(M_H) \quad \text{IV.3}$$

Solving equations [IV.2] and [IV.3], for the charged state z_1 of peak p_1,

$$z_1 = (p_2 - 1)/(p_1 - p_2) \quad \text{IV.4}$$

The charged state z_1 may then be substituted into equation [IV.2] to determine the molecular weight of peptide "P". On the other hand, if a peptide molecule is defined by only one molecular ion peak in the mass spectrum, then there is no straight forward calculation to determine the charged state of the molecular ion, and hence the molecular weight of the peptide. Inspection of the peak profile of molecular ion peaks showed that singly charged molecular ion peaks were associated with a series of isotopic peaks, which are separated from each other by an isotopic spacing of 1 Da (Fig. IV 21 A and B), and doubly charged molecular ion peaks show isotope spacing of 0 5 Da (Fig IV 22 A and B). This suggested that the charged state of a molecular ion peak could be determined by taking the reciprocal of the isotopic spacing of the peak profile. The charged state of a molecular ion peaks determined by taking the reciprocal of the isotopic spacing of the peak profile were verified by the collision induced dissociation mass spectra of the molecular ion. Singly charged molecular ions generated daughter ion mass spectra with mass peaks having lower m/z ratios than that of the parent molecular ion (Fig. IV.21 C), while molecular ions of higher charged state ($z > 1$), generated fragment or daughter ions with m/z values up to "n" times the m/z value of the parent molecular ion, where "n" is the charged state of the parent molecular ion

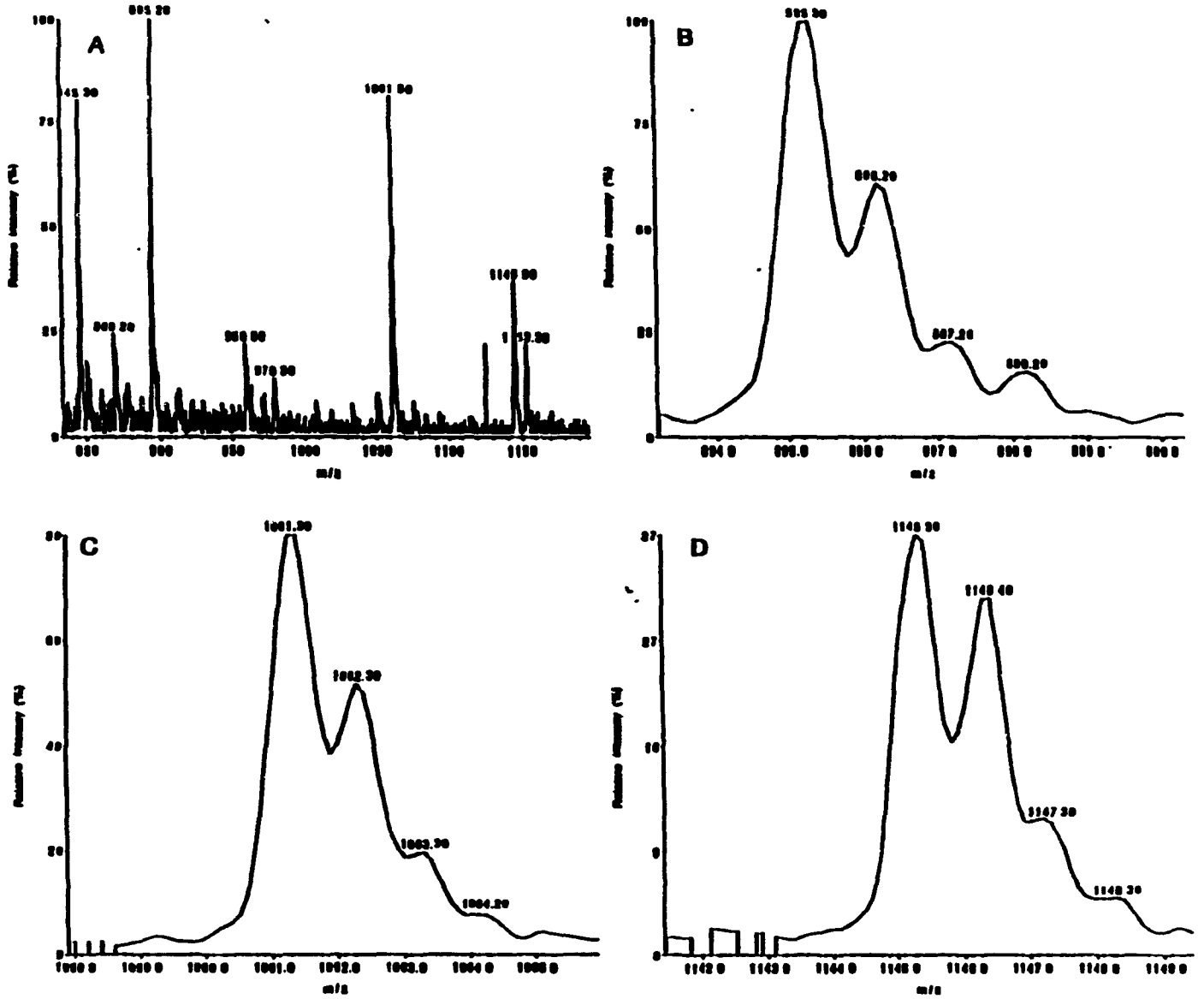

Figure IV.20. "A", shows a typical ESI-mass spectrum of a RP-HPLC fraction of the tryptic hydrolysates of the protein isolates. This represents the ESI/MS spectrum of fraction "7" of the hydrolysate of the crystalline isolate of navy bean after 180 min hydrolysis. B, C, and D show the peak profiles of the peaks at m/z = 895.2, 1061.5, and 1145.3 respectively

(Fig. IV.22 C, where n = 2). The determination of the charged state of molecular ions by use of the isotopic spacing of its mass peak profile provides a simple method for the determination of molecular weights of peptide that are defined by only one molecular ion species in an ESI mass spectra. This technique is useful, since the ESI mass spectrometry of samples containing a mixture of peptide (eg., RP-HPLC fractions of protein hydrolysates) usually produce a mixed mass spectra, containing a mixture of molecular ion series which can complicate charge assignment to mass peaks, and hence, molecular weight determination. Figure IV.20 B, C, and D show the peak profiles of the peaks at m/z 895.20, 1061.50, and 1145.50 in figure IV.20A. All the peaks exhibit isotopic spacing of 1 Da indicating that those molecular ion peaks are singly charged.

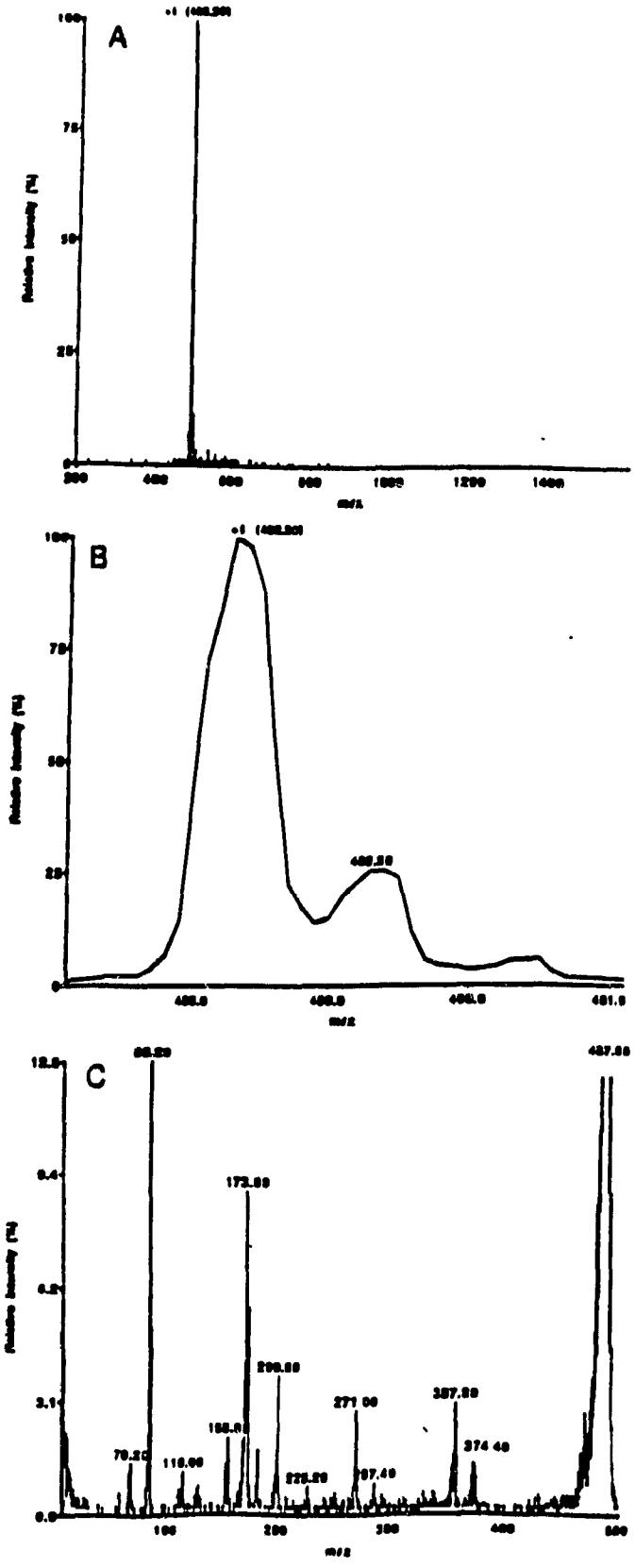

Figure IV.21. "A" shows the ESI/MS spectrum of a singly charged molecular ion (m/z = 488.3). "B" shows the peak profile of the molecular ion in "A", displaying an isotopic spacing of 1 Da, and "C" shows the collision induced dissociation MS daughter ion spectrum of the molecular ion in "A". The daughter ions show peaks with m/z lower than or equal to the parent ion.

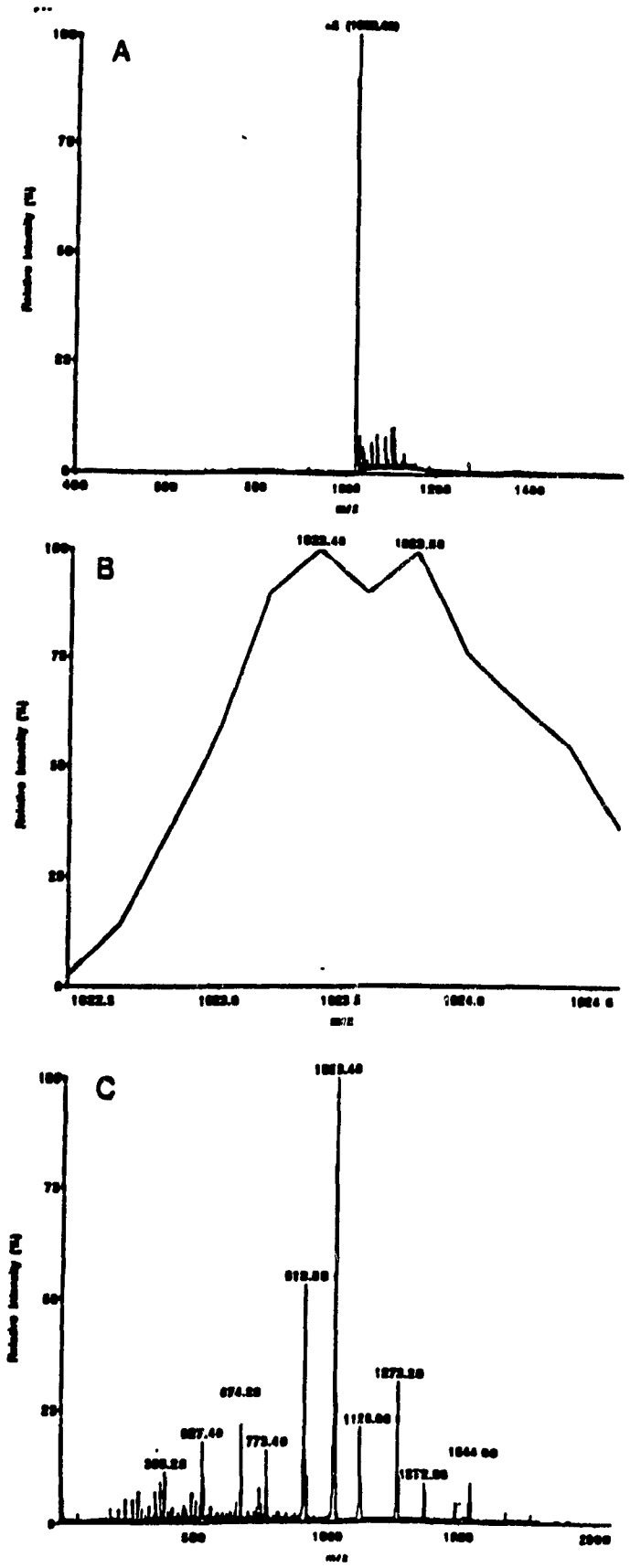

Figure IV.22. "A" shows the ESI/MS spectrum of a doubly charged molecular ion (m/z = 1023.4). "B" shows the peak profile of the molecular ion in "A", displaying an isotopic spacing of 0.5 Da, and "C" shows the collision induced dissociation MS daughter ion spectrum of the molecular ion in "A". The daughter ions show peaks with m/z higher than the parent ion, but less than 2 times the m/z of the parent ion.

IV.E.2. Characteristics of the ESI/Mass Spectra

Low molecular weight peptide (less than 1 k Da) showed abundant singly $(M + H)^+$ charged as well as doubly $(M + 2H)^{+2}$ charged molecular ions (Figure IV.23). As the molecular weight of the peptide increased, the doubly charged molecular ion species $(M + 2H)^{+2}$ showed increasing dominance over the singly charged molecular ion species $(M + H)^+$ (Fig. IV.24). In the case of high molecular weight (MW > 1 kDa) tryptic peptide, the doubly charged molecular ion species $(M + 2H)^{+2}$ completely dominated the ESI mass spectra (Figure IV.25). Some tryptic peptide (Figure IV.26) showed moderately intense triply charged molecular ion $(M + 3H)^{+3}$ species as well as the very intense doubly charged species $(M + 2H)^{+2}$. The observed $(M + 3H)^{+3}$ ion could be due to the presence of internal histidine residue in the tryptic peptide. The dominance of the $(M + 2H)^{+2}$ ion species over the $(M + 3H)^{+3}$ ion species can be attributed to the higher solution basicity of the N-terminal amino group and the C-terminal amino groups of Lysine of arginine (pK_a = 10) compared to the ionizable nitrogen of histidine (pK_a = 7) (Covey et al., 1991).

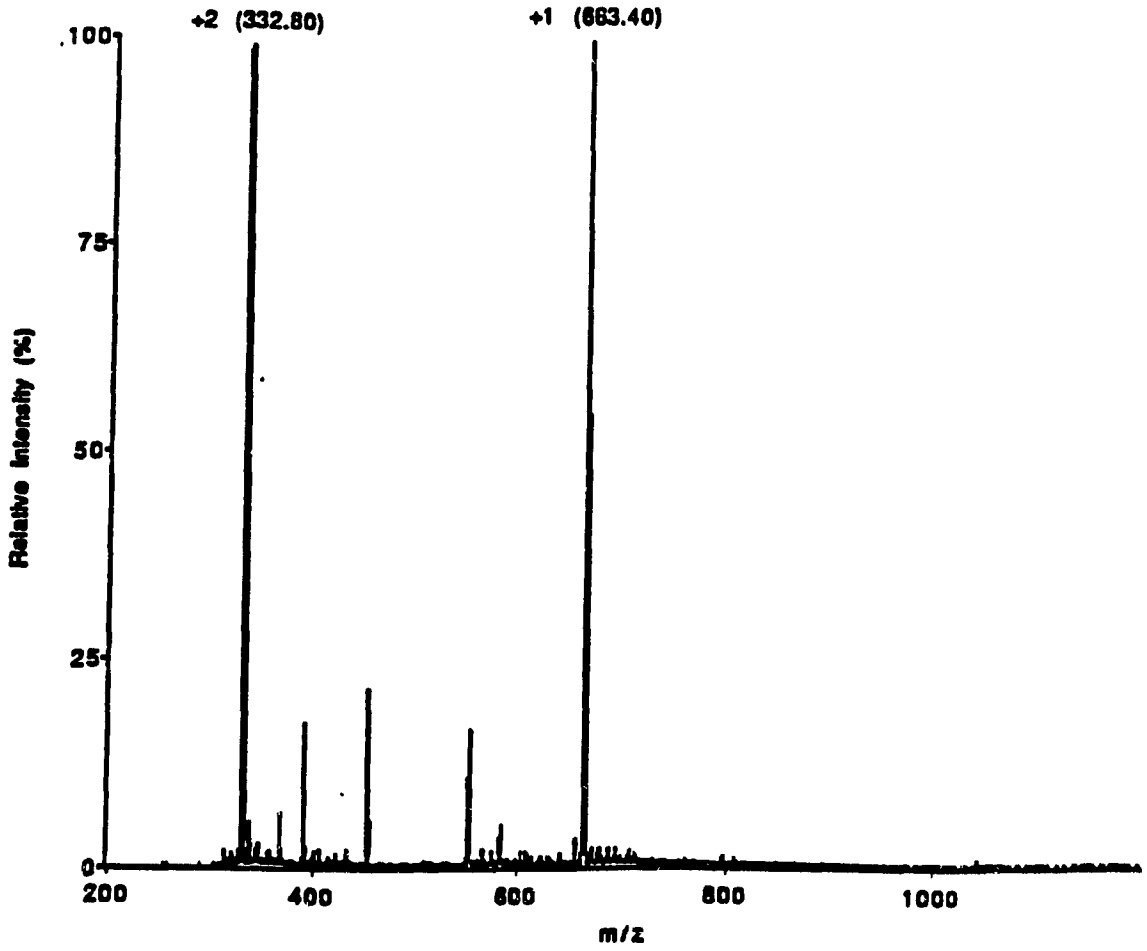

Figure IV.23. The ESI-mass spectrum of a tryptic peptide showing intense singly charged and doubly charged molecular ion species at m/z = 332.8 and 663.4 respectively.

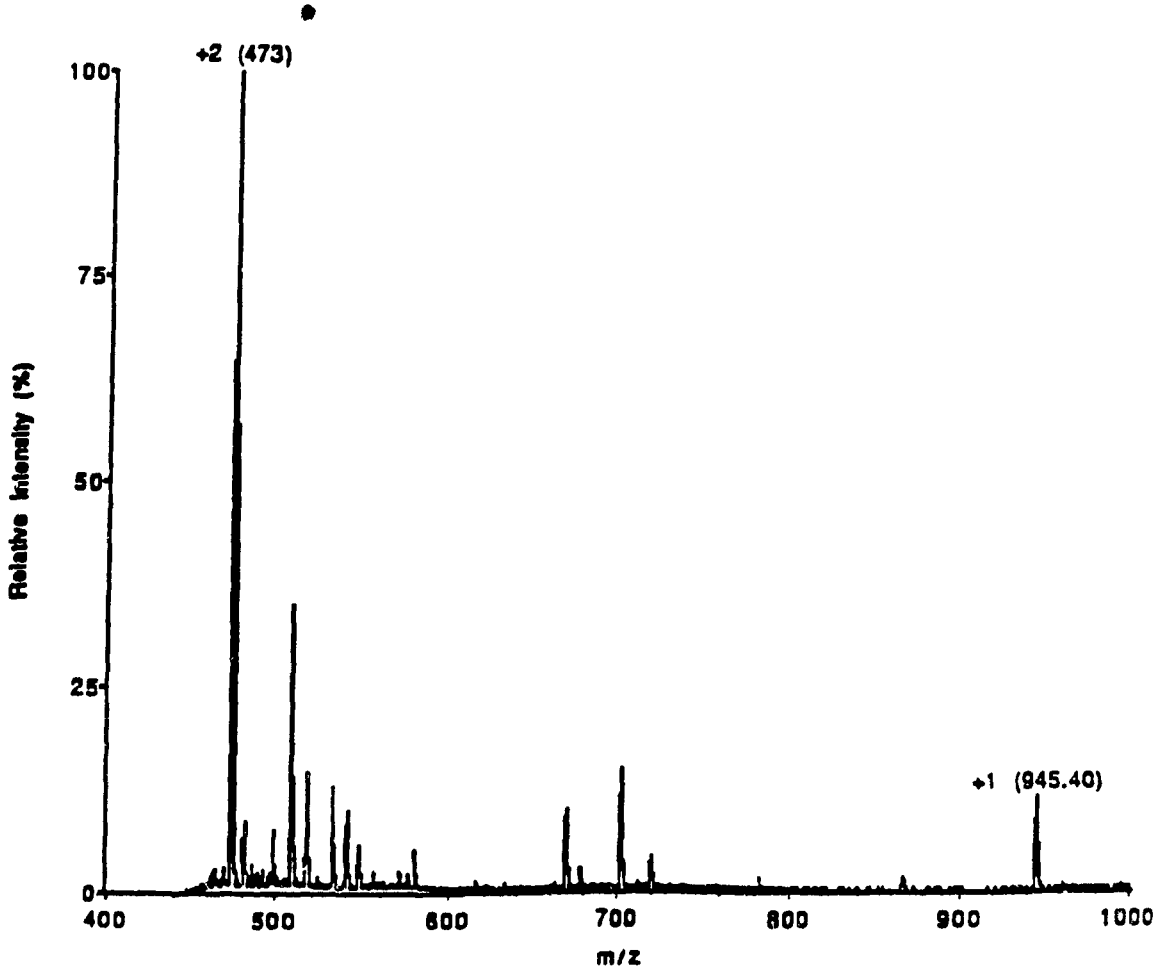

Figure IV.24. The ESI-mass spectrum of a typical tryptic peptide showing singly charged and doubly charged molecular ion species at m/z = 473 and 945.4 respectively, but with the doubly charged specie showing higher intensity.

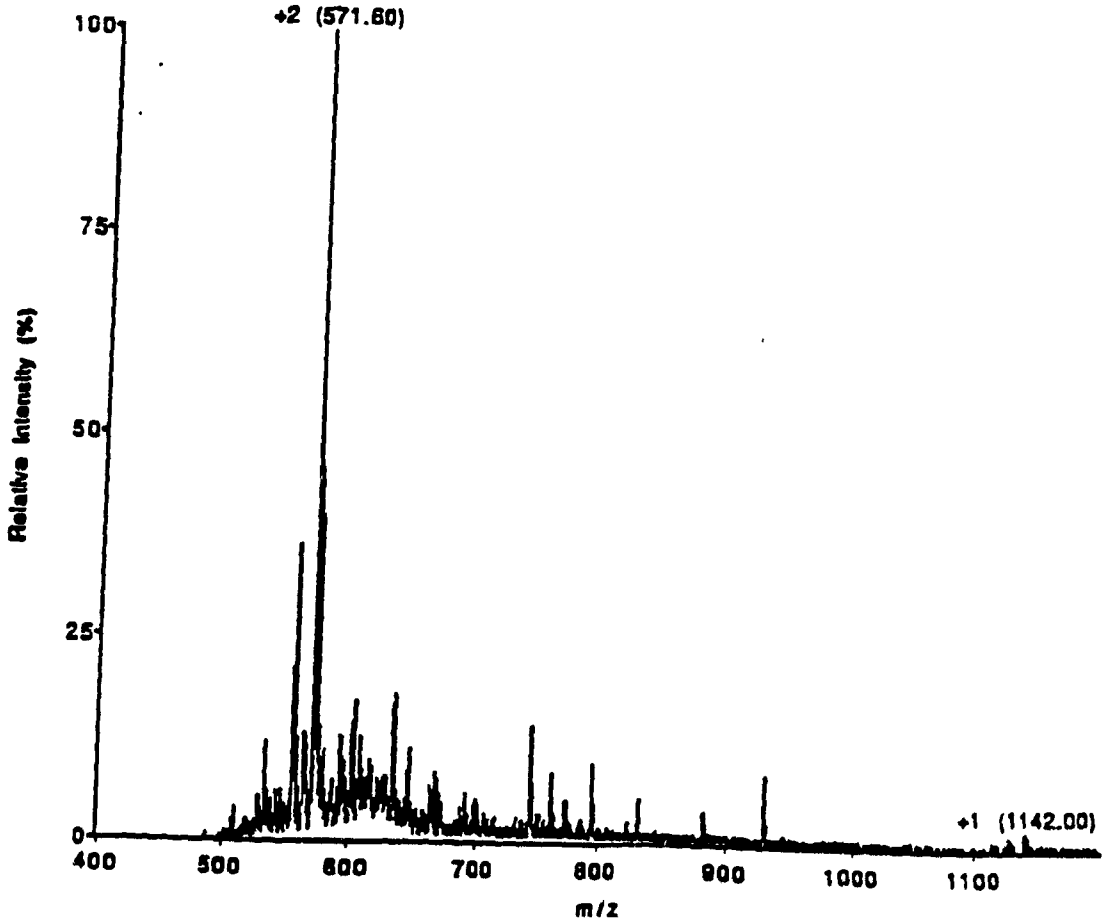

Figure IV.25. The ESI-mass spectrum of a typical tryptic peptide showing singly charged and doubly charged molecular ion species at m/z = 571.6 and 1142 respectively, but with the doubly charged specie completely dominating the spectra.

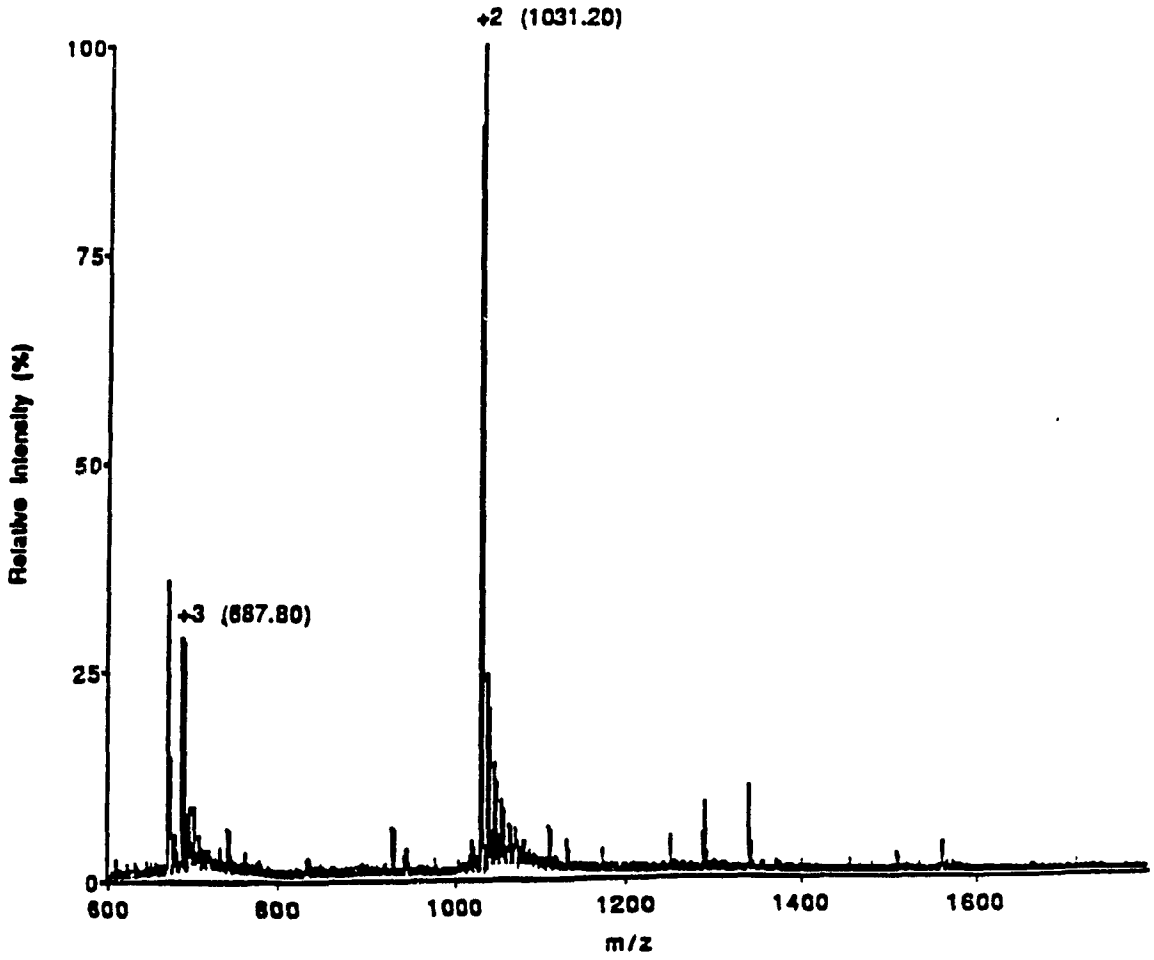

Figure IV.26. The ESI-mass spectrum of a tryptic peptide showing doubly charged and triply charged molecular ion species at m/z = 1031.2 and 867.8 respectively, with the doubly charged specie showing higher intensity than the triply charged specie.

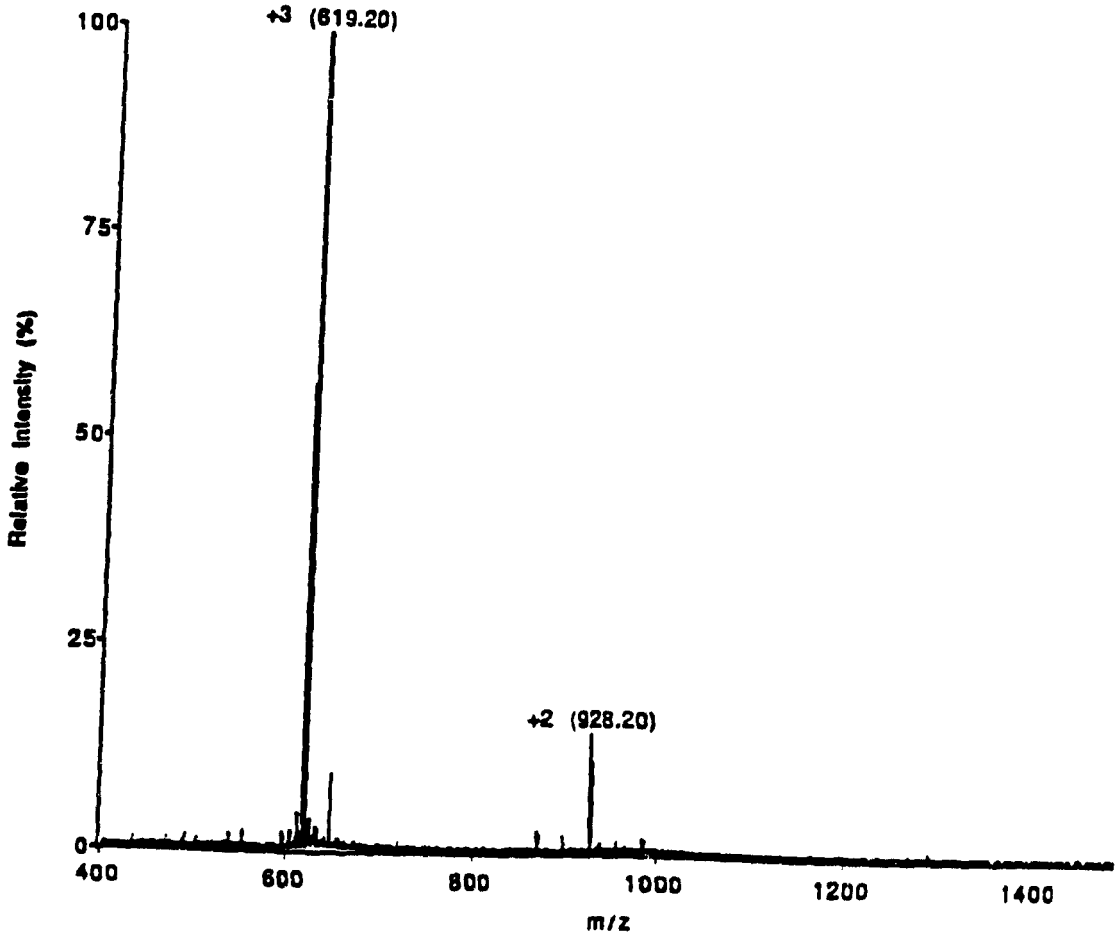

Figure IV.27. The ESI-mass spectrum of a tryptic peptide showing doubly charged and triply charged molecular ion species at m/z = 928.2 and 619.2 respectively, but with the triply charged specie showing higher intensity than the doubly charged specie.

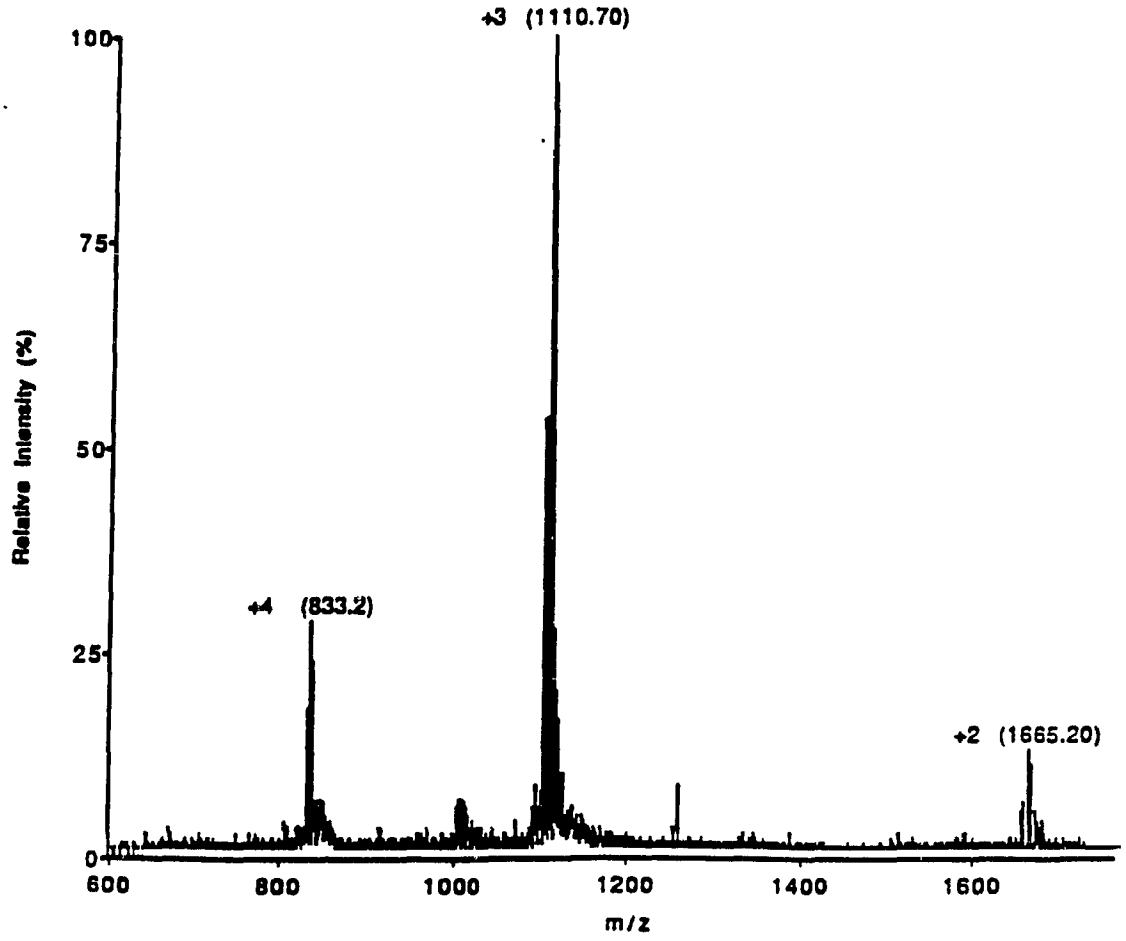

Figure IV.28. The ESI-mass spectrum of a tryptic peptide showing +2, +3, and +4 molecular ion species at m/z = 1665.2, 1110.7, and 833.2 respectively.

Figure IV.27 shows that the $(M + 3H)^{+3}$ molecular ion species of some tryptic peptide showed higher abundance than the $(M + 2H)^{+2}$ molecular ion species. This situation could be due to the presence of internal lysine or arginine residues which are adjacent to a proline residue, and are therefore resistant to tryptic hydrolysis. Figure IV.28 also show that other tryptic peptide generate molecular ion series in which the $(M + 3H)^{+3}$ molecular ion and the $(M + 4H)^{+4}$ molecular ion respectively were predominant over the $(M + 2H)^{+2}$ ion. Peptide that showed such high charged state ions had molecular weights greater than 3 k Da. Tryptic peptide showing intense +3 and +4 charged states that are predominant over the +2 charged state have been reported to be disulphide linked peptide (Covey et al., 1991).

The ESI mass spectra for the RP-HPLC fractions of hydrolysates of crystalline protein isolates showed fewer molecular ion $(M + nH)^{+n}$ peaks than those of their amorphous counterparts. The fewer number of mass peaks in the ESI mass spectra of the RP-HPLC fractions of hydrolysates of the crystalline protein isolates indicates that the RP-HPLC fractions of the hydrolysates of crystalline isolates contained fewer peptide components, and confirms an earlier observation (Section IV.D.1) that hydrolysates of the crystalline isolates were better resolved by RP-HPLC than the hydrolysates of amorphous isolates. In some cases, the ESI mass spectra of RP-HPLC fractions produced only one molecular ion or ion series, showing that those fractions contained only one peptide component, and that, PR-HPLC is capable of resolving complex peptide mixtures

such as protein hydrolysates into individual peptide components in a single run.

The ESI-mass spectra of the reversed phase HPLC fractions of the "abbreviated" peptide pools obtained after partitioning the protein hydrolysates on solid phase extraction cartridges, showed low base line noise and fewer molecular ion peaks when compared with the mass spectra of the RP-HPLC fractions of the un-partitioned hydrolysates. This shows that pre-column partitioning of protein hydrolysates prior to RP-HPLC separation results in high resolution and purer peptide fractions.

Inspection of the ESI-MS spectra of the RP-HPLC fractions of the tryptic hydrolysates of crystalline and amorphous protein isolates from the same bean variety showed that some RP-HPLC fractions of the hydrolysates of the crystalline and amorphous protein isolates from the same bean variety produced quite similar ESI-MS molecular ion profiles. Figure IV.29 A represents the ESI mass spectrum of the RP-HPLC fraction 15 of the hydrolysate of the amorphous isolate of nave bean (ie., peak 10 in figure IV.17B) and figure IV.29B represents the ESI-MS spectrum and fraction 10 of the hydrolysate of the crystalline isolate of nave bean (ie., peak 15 in Figure 16B). Figures IV.30 A and B also represents the ESI-MS spectra of fractions 4 (ie., peak 4 in figure IV.17B) and 7 (ie., peak 7 in figure IV.16B) of the hydrolysates of the amorphous and crystalline isolates of navy beans respectively. The similarity in molecular ion profiles showed by the ESI mass spectra of some RP-HPLC fraction of hydrolysates of crystalline and amorphous protein isolates of the same bean variety suggests that there are

protein components that are common to both crystalline and amorphous isolates derived from the same bean variety. Table IV.14 shows RP-HPLC fractions of hydrolysates of crystalline and amorphous isolates whose ESI-mass spectra showed some similarity in their molecular ion profile. In most cases however, RP-HPLC fractions of hydrolysates of crystalline isolates showed different ESI-mass spectra from those of RP-HPLC fractions of hydrolysates of amorphous isolates from the same bean variety, indicating that there are major compositional differences between crystalline and amorphous protein isolates of the same bean variety, with respect to their protein composition. The molecular weight profile of the major peptide components of hydrolysates of both crystalline and amorphous protein isolates ranged from <200 Da (ie., amino acids) to >5,000 Da.

Table IV.14.

Reversed Phase HPLC fractions of hydrolysates (after 180 min hydrolysis) of crystalline and amorphous isolates from the same bean variety having similar peptide profile.

A. Navy Bean

Crystalline Hydrolysate	Amorphous Hydrolysates
Fraction 6 ---------->	Fraction 3
Fraction 7 ---------->	Fraction 4
Fraction 12 ---------->	Fraction 8
Fraction 15 ---------->	Fraction 10

B. White Kidney Bean

Crystalline hydrolysates	Amorphous Hydrolysates
Fraction 2 ---------->	Fraction 2
Fraction 5 ---------->	Fraction 5
Fraction 7 ---------->	Fraction 8
Fraction 8 ---------->	Fraction 10
Fraction 11 ---------->	Fraction 12

C. Large Lima Bean

Crystalline hydrolysates	Amorphous Hydrolysates
Fraction 2 ---------->	Fraction 2
Fraction 4 ---------->	Fraction 5
Fraction 5 ---------->	Fraction 6
Fraction 6 ---------->	Fraction 7
Fraction 7 ---------->	Fraction 8
Fraction 10 ---------->	Fraction 11

D. Baby Lima Bean

Crystalline hydrolysates	Amorphous Hydrolysates
Fraction 2 ---------->	Fraction 2
Fraction 4 ---------->	Fraction 3
Fraction 5 ---------->	Fraction 7
Fraction 9 ---------->	Fraction 8
Fraction 15 ---------->	Fraction 11

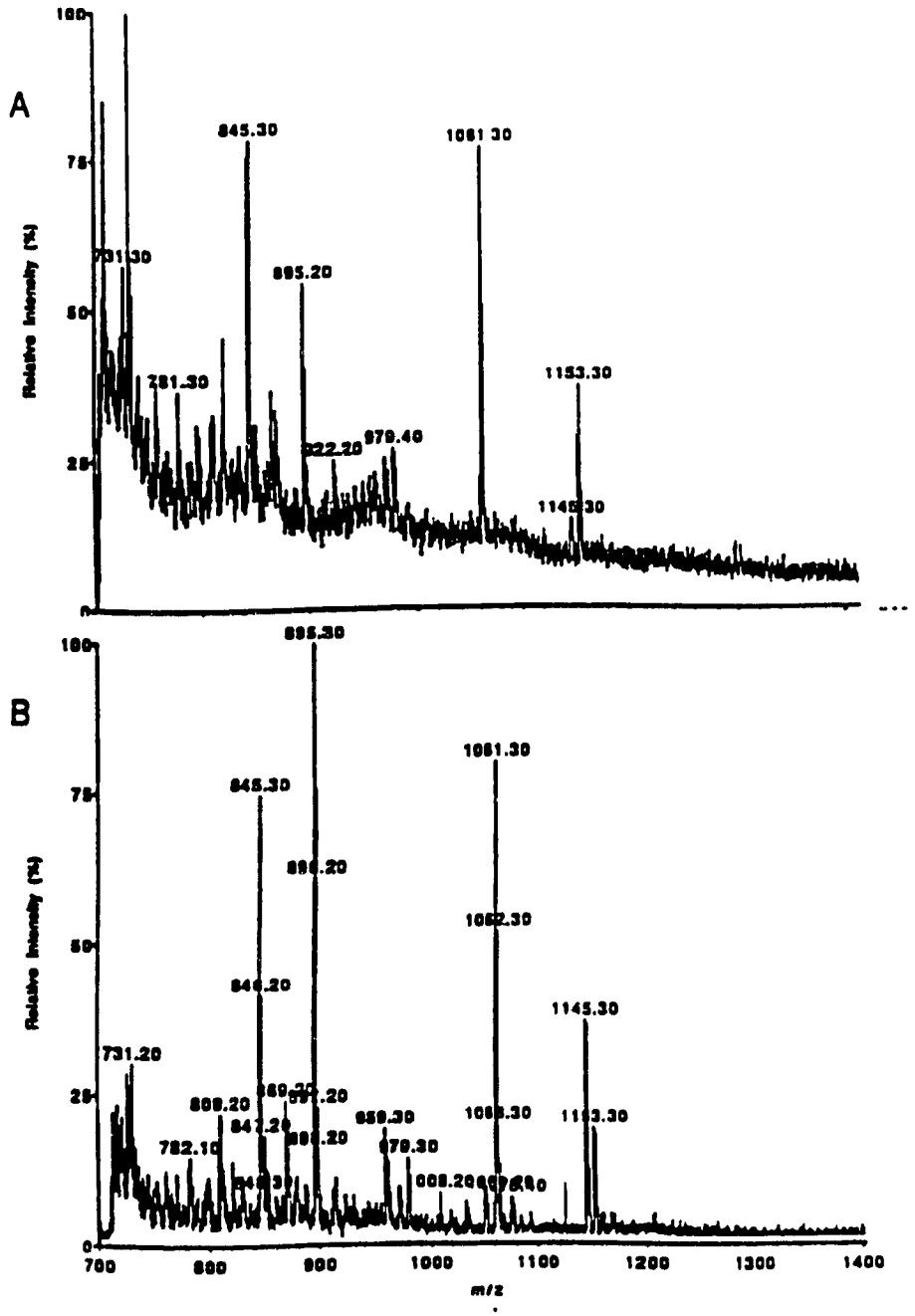

Figure IV.29. ESI-mass spectrum of RP-HPLC fractions of hydrolysates of amorphous and crystalline isolates from the same bean showing quite similar molecular ion profile. "A" shows the ESI-mass spectra of the 4th fraction of the amorphous isolate of navy bean (Fig. IV.17B), and "B" shows the ESI-mass spectra of the 7th fraction of the crystalline isolate (Fig. IV.16B)

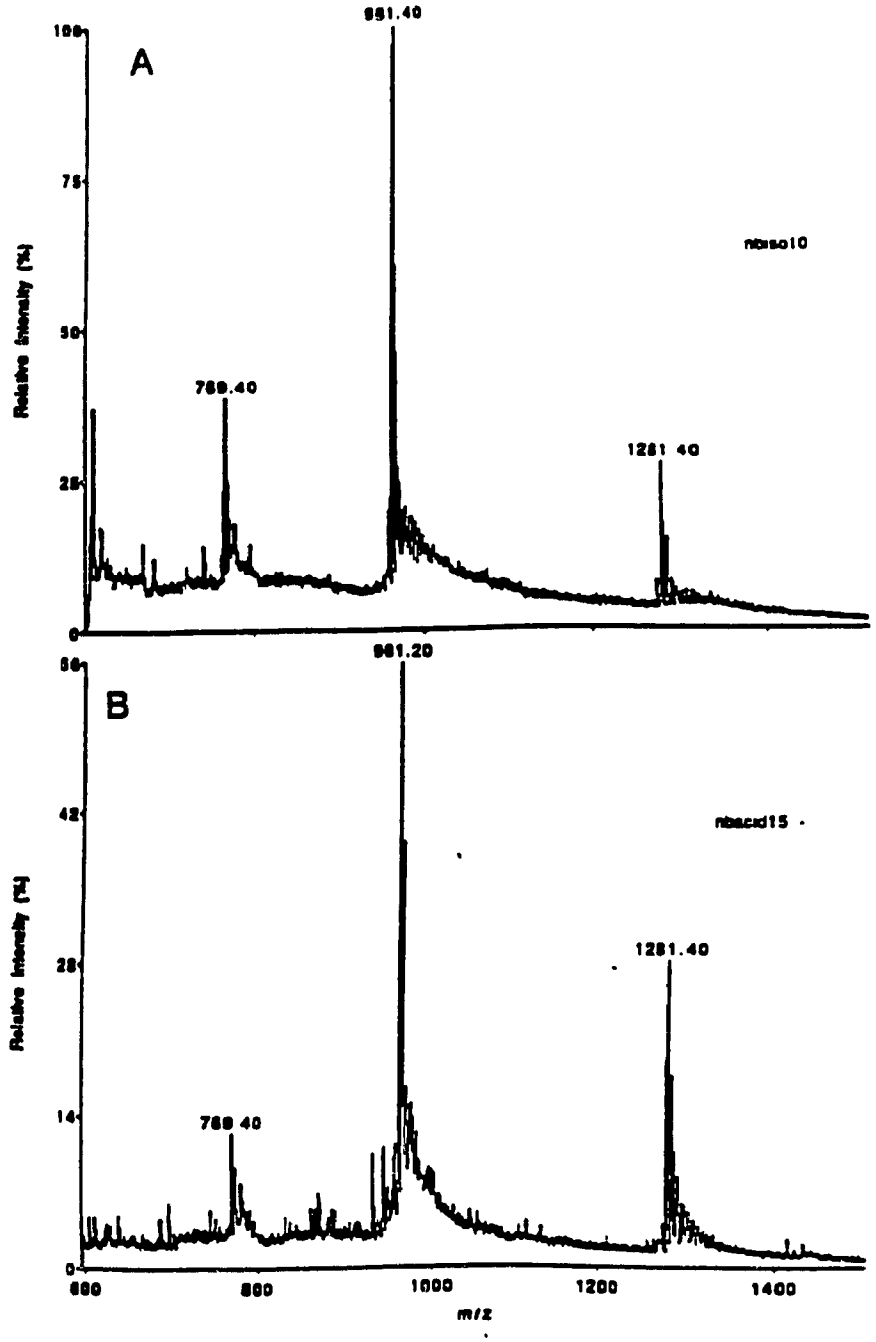

Figure IV.30. ESI-mass spectrum of RP-HPLC fractions of hydrolysates of amorphous and crystalline isolates from the same bean showing identical molecular ion profile. "A" shows the ESI-mass spectra of the 10th fraction of the amorphous isolate of navy bean (Fig. IV.17B), and "B" shows the ESI-mass spectra of the 15th fraction of the crystalline isolate (Fig. IV.16B)

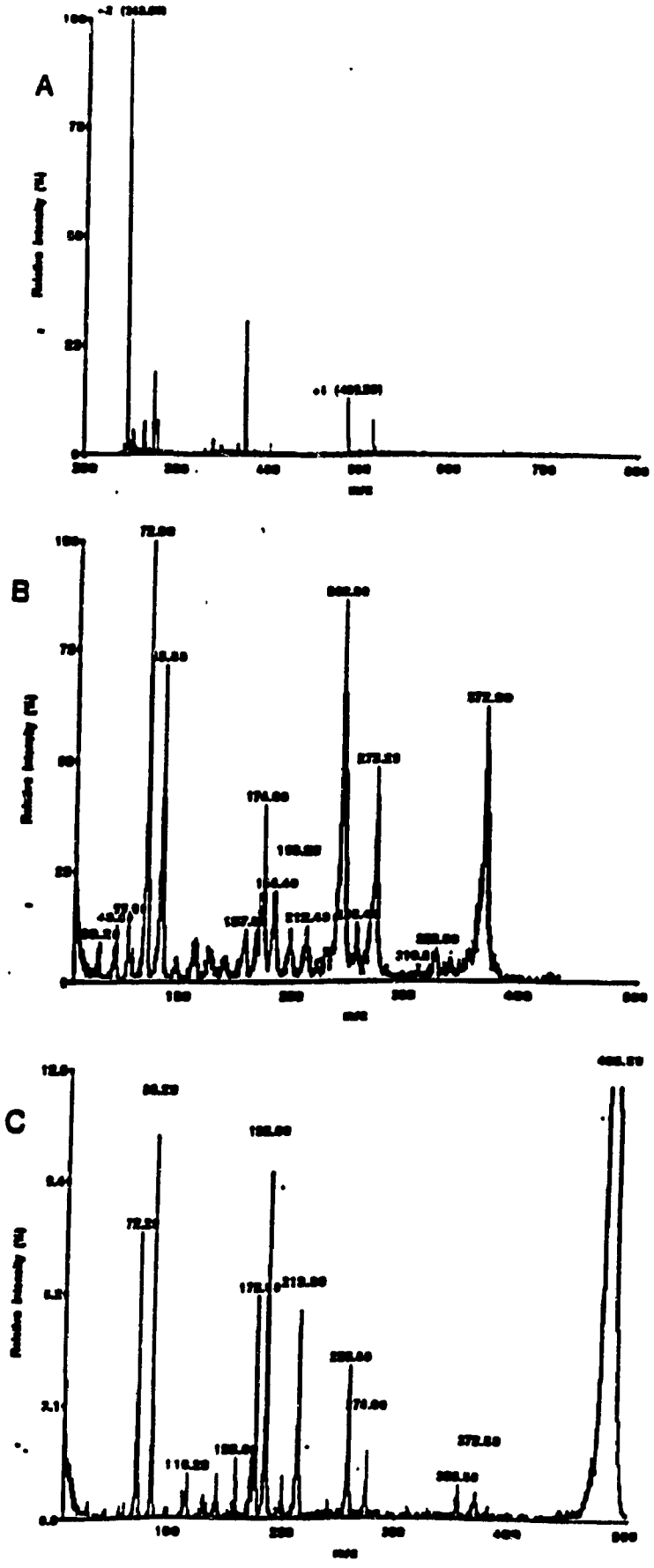

Figure IV.31. "A" shows the ESI-mass spectrum of the RP-HPLC fraction 5 (Peak 4 in figure 16C) showing the singly, and doubly charged molecular ion species of a tryptic peptide at m/z = 243.60 and 486.30 respectively. "B" an "C" show the sequence specific CID daughter ion mass spectra of the parent molecular ions, at m/z = 243.60 and 486.30 respectively.

IV.E.3. Tandem Mass Spectrometry (MS/MS)

The collision induced dissociation (CID) mass spectra of all the selected molecular ions (ESI/MS/CID/MS) were dominated by y_n ion series (daughter ion series formed by the cleavage of the peptide bond along the polypeptide backbone, with charge retention at the C-terminal fragment; providing sequence information from the C- to N-terminal direction), suggesting that the C-terminal amino acid residues of the peptide fractions were basic amino acids (Johnson et al., 1989).

The ESI/MS/CID/MS spectrum in figures IV.31B and IV.C shows the sequence specific daughter ion spectrum of a singly charged state $(M + H)^+$ (m/z = 486.3), and the doubly charged state $(M + 2H)^{+2}$ (m/z = 243.6), of the same peptide molecule (Fig. IV.31A). Both molecular ions show similar fragmentation pattern, confirming that both molecular ion peaks define the same peptide. The $(M + 2H)^{+2}$ ion specie, however, showed a more extensive CID fragmentation, and produced more sequence information than the $(M + H)^+$ ion specie. Peaks at the low mass region of the CID mass spectra (Figures. IV.31B and IV.31C) represent immonium $(H_2N=CHR)^+$ ions of amino acid residues present in the peptide (where - R is the side chain of the amino acid). Peaks at the high mass end of CID mass spectra, formed by the loss of an amino acid side chain may also be diagnostic for the presence of certain amino acid. Their presence is however independent of the position of the amino acid within the peptide (Scoble, 1989). The peaks at m/z = 72, and 85.8 (Figure IV.31C) correspond to the residual mass of the immonium

ions of valine and leucine or isoleucine respectively, and hence indicate the presence of these amino acid within the peptide. Inspection of the daughter ion mass spectra (figures IV.31B and IV.31C) for mass peaks with mass difference corresponding to the monoisotopic masses of the naturally amino acid residues (-NH-CHR-CO-), revealed the y_n ion series y_1 (m/z = 174.6), y_2 (m/z = 273.2), y_3 (m/z = 372) and y_4 (m/z = 485.8) corresponding to the sequence xLeu-Val-Val-Arg (N- to C-terminal direction) were recognized, where xLeu represent leucine or isoleucine residues. The peak at m/z = 43 (Fig. IV.31C), corresponds to the m/z ratio of the isopropyl ion $(CH_3\text{-}CH\text{-}CH_3)^+$, a product of the cleavage of the β-isopropyl group of leucine. The presence of the isopropyl ion suggests that the N-terminal amino acid residue is more likely to be leucine rather than isoleucine. The sequence for the peptide defined by the peaks at m/z 243.6, and 485.8 is therefore Leu-Val-Val-Arg (Figure IV.32). Table IV.16 shows the masses, in atomic mass units (amu), of the 20 physiologically active, naturally occurring α-amino acids in their free state, and within a peptide chain.

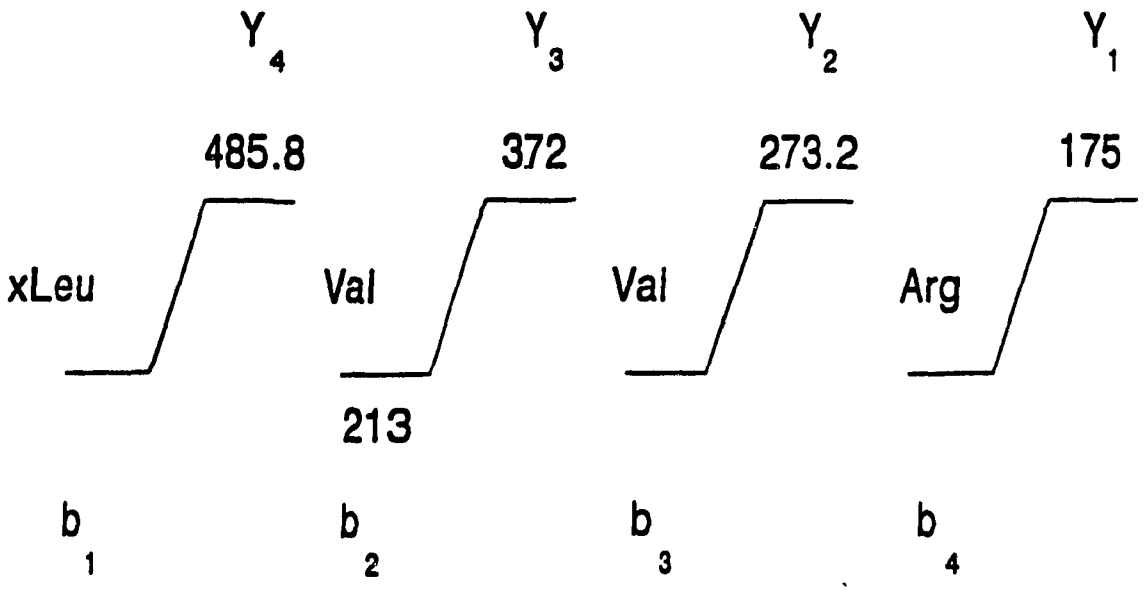

Figure IV.32

Amino acid sequence derived from the sequence specific daughter ion spectra shown in figures IV.31 (B and C) for the peptide defined by the molecular ions at m/z = 243.6 and 486.3, shown in figure IV.31A.

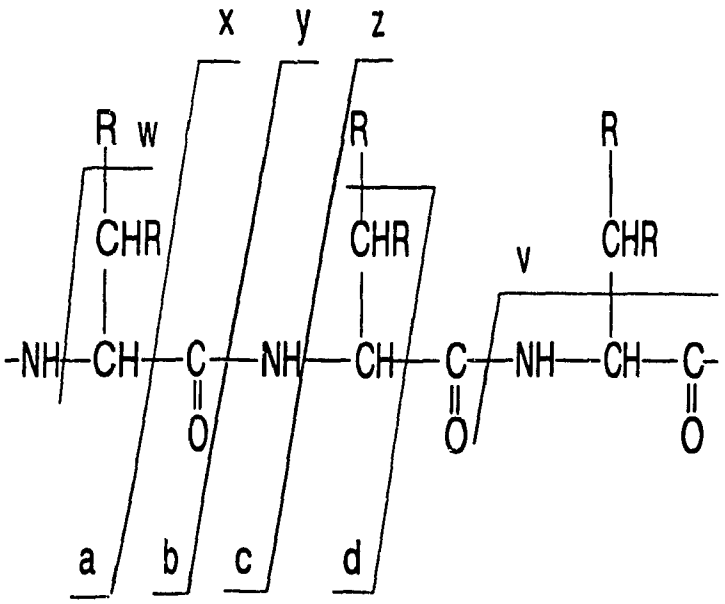

Figure IV.33
Nomenclature of peptide fragments observed in collision induced mass spectrometry of proteins and peptides. Fragmentation series a, b, c, and d represent fragments with charge retention at the N-terminal portion of the fragment, whereas v, w, x, y, and z represent fragments with charge retention at the C-terminal portion of the fragment. The a, b, c, and x, y, z series fragments origimate from direct cleavage of the polypeptide back bone, while d, v, w are series fragments derived from side chain cleavages in addition to back bone cleavages.

IV.E.4. Tryptic Peptide Originating from Phaseolin

Table IV.15 shows the amino acid sequence of tryptic peptides identified as tryptic fragments of α- and β-phaseolin. The results show that tryptic fragments of phaseolin were present in both crystalline and amorphous isolates. Recently, Alli et. al., (1993) have reported the presence phaseolin polypeptides in the crystalline isolate of white kidney bean that is identical to the sequence. The tryptic hydrolysates of navy and white kidney beans (*P. vulgaris*) produced more tryptic fragments of phaseolin than the hydrolysates of baby lima and large lima beans (*P. lunatus*). The low incidence of tryptic fragments of phaseolin in the hydrolysates of *P. lunatus* beans suggests that Phaseolin may probably not be the major protein component of baby lima and large lima beans. A recent report (Alli et al., 1994, in press) suggests that beans of *P. lunatus* may only contain fragments of phaseolin rather that the whole sequence of phaseolin. Although phaseolin is the major protein component of beans of *P. vulgaris* (Deshpande and Damodaran; 1989, 1991a) very few of the tryptic peptide were found to originate from phaseolin, when compared to the number of peptide in the hydrolysates. This can be attributed to the extreme resistance of phaseolin to *in vitro* proteolysis (Deshpande and Damodaran, 1991b).

Table IV.15 (A - G).

Tryptic fragments of Phaseolin identified in the hydrolysates in the MH^+ molecular ion form.

A. Navy Bean Crystalline Isolate

αFragment	βFragment	MH^+ Found	MH^+ Calc	Sequence	Fraction
151 - 154	152 - 155	531.7	532.27	EDLR	(F4)
316 - 320	322 - 326	547.3	547.31	AELSK	(F7)
237 - 241	---	562.3	562.36	KSLSK	(F6)
355 - 360	361 - 366	615.5	615.38	NLLAGK	(F5)
78 - 82	78 - 82	663.3	663.38	LVEFR	(F7)
198 - 203	199 - 204	807.5	807.40	FEEINR	(F3)
70 - 77	70 - 77	1050.3	1050.51	LQNLEDYR	(F10)
361 - 370	367 - 376	1061.3	1061.56	TDNVISSIGR	(F7)
188 - 197	189 - 198	1145.3	1145.60	HILEASFNSK	(F7)
284 - 303	290 - 309	2086.28	2086.19	AIVILVVNEGEAH--VELVGPK	(F10)
316 - 335	322 - 341	2146.28	2146.18	AELSKDDVFVIPA--AYPVAIK	(F12)
81 - 103	83 - 103	2333.38	2333.34	SKPETLLLPQQA--DAELLLVVR	(F14)
204 -225	---	2503.68	2503.26	VLFEEEGQQEGV--IVNIDSEQIK	(F11)

B. Navy Bean Amorphous Isolate

αFragment	βFragment	MH^+ Found	MH^+ Calc	Sequence	Fraction
414 - 415	429 - 430	232.3	232.14	GK	(F1)
355 - 360	361 - 366	615.5	615.38	NLLAGK	(F3)
78 - 82	78 - 82	663.3	663.38	LVEFR	(F4)
70 - 77	70 - 77	1050.3	1050.51	LQNLEDYR	(F3)
361 - 370	367 - 376	1061.3	1061.56	TDNVISSIGR	(F5)
188 - 197	189 - 198	1145.3	1145.60	HILEASFNSK	(F4)
69 - 77	69 - 77	1206.8	1206.62	RLQNLEDYR	(F9)
316 - 335	322 - 341	2146.28	2146.18	AELSKDDVFVIPA--AYPVAIK	(F8)
---	119 - 136	2163.38	2163.00	EYFFLTQGDNPIF--SDNQK	(F6)

C. White Kidney Bean Crystallline

αFragment	βFragment	MH^+ Found	MH^+ Calc	Sequence	Fraction
243 - 243	237 - 237	147.2	147.11	K	(F1)
431 - 431	416 - 416	147.2	147.11	K	(F1)
392 - 395	398 - 401	486.3	487.32	LINK	(F4)
59 - 62	59 - 62	515.3	515.33	VLQR	(F3)
417 - 421	432 - 436	556.0	556.28	GAFVY	(F4)
355 - 360	361 - 366	615.4	615.38	NLLAGK	(F7)
78 - 82	78 - 82	663.2	663.38	LVEFR	(F5)
198 - 203	199 - 204	807.3	807.40	FEEINR	(F7)
316 - 335	322 - 341	2146.28	2146.18	AELSKDDVFVIPA--AYPVAIK	(F8)

D. White Kidney Bean Amorphous Isolate

αFragment	βFragment	MH^+ Found	MH^+ Calc	Sequence	Fraction
355 - 360	361 - 366	615.4	615.38	NLLAGK	(F8)
78 - 82	78 - 82	663.2	663.38	LVEFR	(F5)
198 - 203	199 - 204	807.24	807.40	FEEINR	(F8)

E. Large Lima Bean Crystalline Isolate

αFragment	βFragment	MH^+ Found	MH^+ Calc	Sequence	Fraction
230 - 232	236 - 238	355.2	355.21	HAK	(F1)
233 - 236	239 - 242	436.4	436.22	SSSR	(F1)
392 - 395	398 - 401	488.4	487.32	LINK	(F2)
417 - 421	432 - 436	557.5	556.28	GAFVY	(F2)
52 - 58	52 - 58	887.6	887.45	NQYGHIR	(F2)
70 - 77	70 - 77	1050.7	1050.51	LQNLEDYR	(F4)
361 - 370	367 - 376	1061.7	1061.56	TDNVISSIGR	(F9)
---	310 - 321	1471.7	1472.7	GNKETLEFESYR	(F6)

F. Large Lima Bean Amorphous Isolate

αFragment	βFragment	MH^+ Found	MH^+ Calc	Sequence	Fraction
52 - 58	52 - 58	887.6	887.45	NQYGHIR	(F5)
361 - 370	367 - 376	1060.7	1061.56	TDNVISSIGR	(F10)
188 - 197	189 - 198	1145.7	1145.60	HILEASFNSK	(F10)
---	310 - 321	1473.2	1472.7	GNKETLEFESYR	(F7)
242 - 256	248 - 262	1707.8	1707.79	QDNTIGNEFGN--LTER	(F8)

G. Baby Lima Bean Crystalline Isolate

αFragment	βFragment	MH^+ Found	MH^+ Calc	Sequence	Fraction
392 - 395	398 - 401	488.4	487.32	LINK	(F4)
151 - 154	152 - 155	531.4	532.27	VLQR	(F2)
417 - 421	432 - 436	556.0	556.28	GAFVY	(F4)
78 - 82	78 - 82	663.2	663.38	LVEFR	(F5)
70 - 77	70 - 77	1050.7	1050.51	LQNLEDYR	(F7)

H. Baby Lima Bean Amorphous Isolate

αFragment	βFragment	MH^+ Found	MH^+ Calc	Sequence	Fraction
392 - 395	398 - 401	486.4	487.32	LINK	(F3)
417 - 421	432 - 436	556.0	556.28	GAFVY	(F3)
78 - 82	78 - 82	663.2	663.38	LVEFR	(F7)
52 - 58	52 - 58	888.18	887.45	NQYGHIR	(F3)
188 - 197	189 - 198	1144.7	1145.60	HILEASFNSK	(F7)

CONCLUSION

1). Dilute sodium hydroxide was a more effective solvent than citric acid solutions for solubilizing legume seed proteins, but the method of isoelectric precipitation used to recover dissolved protein in sodium hydroxide extraction results in the entrapment of extraneous material in the protein matrix, reducing the purity of the protein isolate.

2). The determination of degree of hydrolysis by the TCA solubility index method and the formol titration method coupled reversed phase h.p.l.c provided a very clear picture of the hydrolytic process.

3). The formol titration results revealed that there is no clear relationship could be established between the microstructure of the bean protein isolates and the susceptibility of their constituent proteins to tryptic hydrolysis.

4). The crystalline isolates of both *P. vulgaris* and *P. lunatus* beans produced more soluble hydrolytic products than the amorphous isolates due probably to a higher content of albumins in the crystalline isolates.

5). Limited tryptic hydrolysis (30 to 45 min hydrolysis) improved the emulsifying and foaming properties of the protein isolates and casein, while extensive hydrolysis (after 90 min hydrolysis) reduced their functional properties.

6). The protein isolates and their corresponding hydrolysates exhibited superior foaming properties than casein and its hydrolysates. Casein on the other hand showed better emulsifying properties than the bean isolates, with the exception of WKB(crys) isolate. The results show that emulsifying and foaming properties of proteins and peptides depend on different physico-chemical characteristics.

7). Pre-HPLC partitioning of protein hydrolysates into acidic, basic and neutral peptide pools, using the Sep-Pak Plus solid phase extraction cartridges produced less complex and 'cleaner' peptide mixtures which were better resolved by reversed phase HPLC (Spanier and Edwards, 1987; Voirin and Letavernier, 1991).

8). The reversed phase peptide maps of the hydrolysates can be used to differentiate crystalline and amorphous isolates from the same bean type, or from different origins. Progressive peptide mapping of the hydrolysates with time can be used to qualitatively and quantitatively follow the sequential cleavage of the polypeptide backbone of proteins during the hydrolytic process, and hence characterize their tertiary and quaternary structure.

9). A combination of ESI/MS and ESI/MS/MS of Reversed phase HPLC fractions of protein hydrolysates provides a simple and efficient means for the structural characterization of proteinawand peptides.

VI. RECOMMENDATIONS

A). Further structural studies in this direction should be done on purified protein fractions of the bean isolates, as the presence of other protein complicates the interpretation and quantitative analysis of the chromatogram of the protein hydrolysate.

B). Further work should be done to ascertain the individual protein components and composition of the crystalline amorphous bean isolates.

C). Further functional studies should be done on the protein isolates to establish appropriate conditions of modification at both lab and pilot plant levels, as the bean isolates show very promising functional properties that can be harnessed for the food industry.

REFERENCES

Adler-Nissen, J., Eriksen, S., Olsen, H. S. (1983). Improvement of the functionality of vegetable proteins by controlled enzymatic hydrolysis. Plant Foods. Hum. Nutr. **32,** 411 - 423.

Adler-Nissen, J. (1982). Limited enzymatic degradation of proteins: A new approach in the industrial application of hydrolysis. J. Chem Tech. Biotech. **32,** 138- 156.

Adler-Nissen, J. (1979). Determination of the degree of hydrolysis of food protein hydrolysates by Trinitrobenzene sulfonic acid. J. Food Chem. **27,** 1256 - 1262.

Ahnoff, M., Grundecik, I., Arfwidsson, A., Fonselius, I., and Persson, B. A. (1981). Anal. Chem. **53**: 485 - 489.

Akeson, W. R., and Stahmann. (1964). A pepsin pancreatic digest index of protein quality evaluation . J. Nutr. **83**: 257.

Alli, I , Gibbs, B. F., Okoniewska, M. K., Konishi, Y., Dumas, F., (1993). Identification and characterization of phaseolin polypeptides in a crystalline protein isolate from whote kidney bean (*Phaseolus vulgaris*). J. Agric. Food Chem. **41**: 1830 - 1834.

Alli, I., Gibbs, B. F., Okoniewska, M. K., Dumas, F., Konishi, Y., (1994). Identification of phaseolin fragments in a crystalline protein isolate from large lima beans (*Phaseolus lunatus*). Biochem. et Biophysica. Acta. In press.

Allison, L. A., Mayer, G. S., Shoup, R. E., (1984). OPA Derivatization of Amines for High Speed Liquid Chromatography / Eletrochemistry. Anal. Chem. **56**: 1089 - 1096.

Andrew, P. C. (1990). Selective isolation of disulfide containing peptides from trypsin digest using Strong cation exchange HPLC. "In Current Research in protein Chemistry: Techniques, Structure, and Function." (J. J. Villafranca. ed). Academic press Inc. San Diego. p 95- 101

Arai, S., and Fujimaki, M. (1987). Enzymatic modification of proteins with special reference to improving their functional properties. In Food Enzymology. 1987. vol 2. (P. F. Fox, ed). p. 83 -104. Elsevier Sci. Pub.

Barber, M., Bordoli, R. S., Sedgwick, R. D., Tyler, A. N. (1981) Fast Atom Bombardment of Solids: A New Ion Source for Mass Spectrometry. Chem Soc. Comm. **6** 325.

Bean, M. F., Carr, S. A. (1991). Tandem Mass Spectrometry of Peptides Using Hybrid and Four Sector Istruments: A Comparative Study. Anal Chem. **63,** 1473 - 1481.

Bergenstahl, B. A., and Claesson, P. M. (1990). Surface forces in emulsions. In Food Emulsions. Kare Larsson and Stig, E. Friberg (ed). p. 41. Marcel Dekker Inc. New York.

Bidlingmeyer, B. A., Cohen, S. A., Tarvin, T. L., (1984). Rapid Analysis of Amino Acid Using Precolumn Derivatization . J. Chromatogr. **336**: 93 - 104.

Biemann, K. (1986). Mass Spectrometric Methods for Protein Sequencing. Anal Chem. **58** 1288A

Bohlen, P., Mellet, w, (1979). Automated Fluorescamine Amino Acid Analysis: The Determination of Proline and Hydroxyproline. Anal. Biochem. **94**: 313 - 321.

Boppana, V. K., Miller-Stein, C., Politowski, J. F., Rhodes, G. R. (1991). High Performance Liquid Chromatogrphic Determination of peptides in Biological Fluids by Automated Precolumn Fluorescence Derivatization with Fluorescamine. J. Chromatogr. **545**: 319 - 327.

Britten, M., and Lavoie, L. (1990). mesure de la fermente des mousses: Effet du temps de drainage. Brief communications and abstracts of rosters of the XXIII international dairy congress, Montreal, Canada. 258.

Britten, M., and Lavoie, L. (1992). Foaming properties of proteins as affected by concentration. J. Food Sci. **57**: 1219 - 1222.

Brown, C. A., Bennett, H. P. J., Solomon, S. (1982). The Isolation of Peptides by High Performance Liquid Chromatography Using Predicted Elution Position. Anal. Biochem. **124**, 201 - 208.

Burgoyne, R., Stacey, C., Young, P., Astephen, N., Merion, M. (1989). Analysis of peptides at low levels using chromatographic systems optimized for narrow bore columns. "In Tecniques in Protein Chemistry. "(T. E. Hugli. ed). Academic Press Inc. p 399- 416.

Cachia, P. J., van Eyk, J., Chong, P. C. S., Taneja, K., Hodges, R. S. (1983). Separation of Basic Peptides by Cation Exchange High Performance Liquid Chromatography. J Chromatogr. **266**, 651 - 659.

Cady, N. D., Carter, A. E., Kayne, B. E., Zabik, M. E., and Uebersay, M. A. (1987). Navy bean flour substitution in a master mix used for muffins and cookies. Cereal Chem. **64**: 193.

Cambero, M. I., Seuss, I., Honikel, K. O., (1992). Flavour Compounds of Beef Broth as Affected by Cooking Temperature. J. Food Sci. **57** (6) 1284.

Cappiello, A., and Bruner, F. (1993). Micro flow particle beam interface for capillary liquid chromatography / mass spectrometry. Anal Chem. **65**: 1281 - 1287.

Caprioli, R. M., Fan, T., and Cottrell, J. S., (1986). Continuous Flow Sample Probe for Fast Atom Bombardment Mass Spectrometry. Anal. Chem. **58**: 2941

Casella, M. L. A., and Whitaker, J. R. (1990). Enzymatically and chemically modified zein for improvement of functional properties. J. Food Biochem. **14**: 453 -475.

Champion, H. M. and Stanley, D. W. (1982). High Performance Liquid Chromatographic Separation of Peptides of Bitter Cheddar Cheese. Food Sci. Technol. **15**: (4). 283 - 288.

Chang, K. C., and Saterlee, L. D. (1981). Isolation and characterization of the major proteins from great northern beans (*Phaseolus vulgaris*). J. Food Sci. **46**: 1368.

Charbonneau, H. (1989). Strategies for Obtaining Partial Amino Acid Sequence Data from small quantities (<5nmol) of pure or partially purified protein. "In A practical Guide to protein and peptide purification for microsequencing". (P. T. matsudaira. ed). Academic Press Inc. San Diego.

Chavan, J. K. and Kadam, S. S. (1993). Nutritional enrichment of bakery products by supplementation with nonwheat flours. CRC Rev. Food Sci. Nutr. **33**(3): 189.

Chobert, J. M., Bertrand-Harb, C., Dalgalarrondo, M., Nicholas, M. G. (1989). Solubility and Emulsifying Properties of beta Casein Modified Enzymatically by trypsin. J. Food Biochem. **13**: 335 - 352.

Chobert, J. M., Bertrand-Harb, C. and Nicholas, M-G. (1988). Solubility and Emulsifying Properties of Casein and Whey Proteins Modified by Trypsin. J. Agric Food Chem. **36**: 883 - 892.

Clark, D. C., Mingius, J., Sloan, E. F., Smith, J. L. (1987). The Formation and Break Down of Protein Stabilized Foams. "In Food Emulsions and Foams". (Ed. Dickinson, E.). Royal Soc. Chem. **55**:

Clegg, K. M., Lee, Y. k., McGilligan, J. F. (1982). Technical note: Trinitrobenzene sulfonic acid and Ninhydrin reagents for the assessment of protein degradation in cheese samples. J. Food Technol. **17,** 517 -520.

Colbert, B. L., Decker, E. A. (1991). Antioxidant Activity of an Ultra Filtration Permeate from Acid Whey. J. Food Sci. **56**: (5) 1248.

Colilla, F. J., Yadav, S. P., Brew, K., Mendez, E., (1991). Peptide Maps at Picomole Level obtained by Reversed Phase High Performance Liquid Chromatography and Derivatization with Phenylisothiocyanate. Microsequencing of Peptides. J. Chromartogr. **548**: 303 - 310.

Cordle, C. T., Mahmoud, M. I., and Moore, V. (1991). Immunogenecity evaluation of protein hydrolysates for hypoallergenic infant formulae. J. Pediatr. Gastroenterol Nutr. **13**: 270 - 276.

Covey, T. R., Huang, E. C., Henion, J. D., (1991). Structural Characterization of Protein Tryptic Digest via Liquid Chromatography Mass Spectrometry and Collision Induced Dissociation of their Doubly Charged Molecular Ion. Anal Chem. **63**: 1193 - 1200.

Cuthbertson, D.P. (1950). Amino acids and protein hydrolysate in human and animal nutrition. J. Sci.Food Agric. **1,** 35 -41

Dagorn-Scaviner, C., Gueguen, J., and Lefebvre, J. 1987. Emulsifying properties of pea globulins as related to their adsorption behaviour. J. Food Sci. **52**: 335.

Das, K. P., and Kinsella, J. E. 1990. Stability of food emulsions: Physicochemical role of protein and non-protein emulsifiers. Adv. Food Nutr. Res. **34**: 81.

Deeslie, W. D. and Cheryan, M. (1988). Functional properties of soy protein hydrolysates from a continuous ultra filtration reactor. J. Agric Food Chem. **36**: 26 -31.

Deshpande, S. S. and Damodaran. S., (1991a). Legumes, in foods of plant origin: Production, technology and human nutrition. (Salunkhe, D. K. and Deshpande, S. S., Eds). Van Nostrand Reihold, New York. 137.

Deshpande, S. S., and Damodaran, S (1991b). Denaturation behaviour of phaseolin in urea, guanidine HCl and SDS solutions. J. Protein Chemistry. **10**: 103.

Deshpande, S. S., and Damodaran, S (1990). Conformational characteristics of legume 7S. globulins as revealed by circular dichroic derivative UV absorption and fluorescence techniques. Int. J. Peptide Research. **35**: 25.

Deshpande, S. S., and Damodaran, S. (1989). Heat induced conformational changes in phaseolin and its relation to proteolysis. Biochem. et Biophys. Acta. **998**: 179 - 188.

Deshpande, S. S and Nielsen, S. (1987). Nitrogenious constituents of selected grain legumes. J. Food Sci. **52**: 1321.

Deshpande, S. S. (1993). Food legumes in human nutrition: A personal perspective. CRC. Critical Rev. Food Sci. Nutr. **32**(4): 333 - 363.

Dhurandhar, N. V., and Chang, K. C., (1990). Effect of cooking on firmness, trypsin inhibitors, lectins and cystine/cystienecontent of navy and red kidney beans. J. Food Sci.

Di Lollo, A. B. (1990). Thermal and surface properties of crystalline and non-crystalline legume seed proteins. MSc. Thesis, Dept. Food Sci. & Agric Chem. McGill University.

Dickinson, E., and Stainsby, G. (1982). Rheology. In colloids in Foods. Applied Food Science Publishers, London, UK.

Dickinson, E., and Wosket, C. M. (1989). Competitive adsorption between proteins and small molecule surfactants in food emulsions. In Food Colloids. (R. D. Bee, P. Raymond, and J. Mingins. eds) 1989. Royal Soc Chem. p.74.

Dizdaroglu, M. (1985). Weak Ion Exchange high Performance Liquid Chromatography of Peptides. J. Chromatogr. **334**, 49 - 69.

Dreher, M. L., and Paket, J. W. (1984). Effects of supplementtation of short bread cookies with roasted whole navy bean flour and high protein flour, J. Food Sci. **49**: 922.

Edwards, J. H., Shipe, W. F. (1978). Characterization of plastein reaction products formed by pepsin, and chymotrypsin and papain treatment of egg albumen hydrolysates. J. Food Science. **43.** 1215- 1218.

Eicher, N. J., and Satterlee, L. D. (1988). Nutritional quality of great northern bean proteins processed at varying pH. J. Food Sci. **53**: 1139.

Elkin, R. G., Wusynczuk, A. M., (1988). Amino acid analysis of foodstuff hydrolysate by precolumn derivatization with phenylisothiocyanate and reversed phase HPLC. Cereal Chemistry. **64** (4). 226 - 229.

Estevez, A. M., and Luh, B. S., (1985). Chemical and physical characteristics of ready -to-eat dry beans. J. Food Sci. **50**: 777

Fan, T. Y., and Sosulski, F. W. 1974. Dispersibility and isolation of proteins from legume flours. J. Can Inst Food sci. Technol. **7**: 256 - 259.

Feng, R., Konishi, Y., and Bell, A, W., (1991). High accuracy molecular weight determination and variation characterization of protein up to 80 Ku by ionspray mass spectrometry. J. Am. Soc Mass Spectrom. **2**: 387 - 410.

Feeney, R. E , Yamasaki, R. B. and Geoghegan, K. F. (1982). "In Modification of Proteins: Food Science and Pharmacological Aspects" (Ed. R. E. Feeney and J. R. Whitaker) Advances in Chemistry Series. 198. Am. Chem. Soc. Washington D.C. USA. pp. 3 - 35.

Finney, P. L., Beguim, D., and Hubbard, J. D. (1982) Effects of germination on bread baking properties of mung bean (*Phaseolus aureus*) and garbanzo bean (*Cicer arietinum*). Cereal Chem., **59**: 520.

Fox, P. F., Murrissey, P. A. and Mulvihill, D. M. (1982). Chemical and Enzymatic Modification of Food Proteins. "In Developments in Food Proteins". Vol. 1. (Ed. B. J. F. Hudson) Applied Science Pub. London England.

Frenz, J., Bourell, J., Hancock, W. S., (1990). High Performance Displacement Chromatography Mass Spectrometry of Peptides of recombinant Human Growth Hormone. J. Chromatogr. **512**: 299 - 314.

Friberg, S. E., Goubran, R. F., and Kayali, I. H. 1990. Emulsion stability. In Food Emulsions. Kare Larsson and Stig, E. Friberg (ed). p. 1. Marcel Dekker Inc. New York.

Ganapathy, V. and Leibach, F. (1985). Is Intestinal Peptide Transport Energized by a Proton Gradient. Am. J. Physiol. **249**: G153.

Garcia-Chacon, E. J., Satterlee, L. D., and Hanna, M. A. (1990). Heat induced gells from partially hydrolyzed soy protein isolate. J. Food Biochem. **14**: 15.

Gardner, M. L. G. (1984). Intestinal Assimilation of Intact Peptides and Proteins from the Diet- a Neglected Field?. Biol. Rev 59: 289.

Gauthier, S. F., Vachon,C., Jones, J. D., and Savoie, L. (1982). Assessment of protein digestibility by in vitro emzymatic hydrolysis with simultaneous dialysis. J. Nutr. **112**: 1718.

Gmoshinskii, I. V., Kruglik, V. I., Samenkova, N. F , Krzhechkovskaya, V. V., and Zorin, S. N. (1991). Characterization of peptide preparations obtained after enzymatic hydrolysis and ultrafiltration of milk proteins intended for utilization in specialized nutrition formulae. Voprosy Pitaniya. 0(3): 21 (Abstr).

Grego, B., Hearn, M. T. W., (1983). High Performance Liquid Chromatography of Amino Acids, Peptides and Proteins.
XXXIX' Reversed Phase High Performance Liquid Chromatographic Analysis of DNS Amino Acid Comparison Of Different Elution Systems. J. Chromatogr. **255**: 67 - 77.

Grego, B., Nice, E. C., Simpson, R. J. (1986). Use of Scanning Diode Array Detector with Reversed Phase Microbore Columns for Real Time Spectral Analysis of Aromatic Amino Acidsin peptides at Submicrograme Levels: Application to Peptides and Protein Microsequencing. J. Chromatogr. **352**: 359 - 368.

Grimble, G. K., Keohane, P. P., Higgins, B. E., Kaminski, M. V., and Silk, D. B. A. (1986). Effect of peptide chain length on amino acid nitrogen absorption from lactalbumin hydrolysates in the normal human jejunum. Clinical Sci. **71**: 65 - 69.

Grimble, G. K., Rees, R. G., Keohane, P. P., Cartwright, T., Desreumaux, M., Silk, D. A. B. (1987). Effect of Peptide Chain Length on absorption of egg proyein hydrolysates in normal human Jejunum. Gastroenterlogy. **92**, 136 -142.

Hail, M., Lewis, S., Zhou, H., Jardin, I., Whitehouse, C. (1990). Analysis of Peptides and Proteins by Tandem Quadrupole Mass Spectrmetry. "In Current Research in Protein Chemistry: Tecniques, Structure, and Function." (J. J. Villafranca. ed) Academic Press. p 105.

Haque Z. U. (1993). Influence of milk peptide in determining the functionality of milk proteins. J. Dairy Sci. **76**: 313 - 320.

Harwalkar, V. R. and McMahon, D. J. (1993). Biological and Food Functional Characteristics of Milk Protein Hydrolysis Products (Introduction). J. Dairy Sci. **76**: 300.

Hesseltine, C. W. (1979). Some important fermented foods of Mid-Asia, the midle East, and Africa. J. Am. Oil chemist Soc. **56.** 367 -374.

Hiller, E. (1986). Simplified Peak Identification and Confirmation with a New Easy to Use UV-VIS Photodiode Array Detector. J. Analysis and Purification. March 1986. pp.22.

Holcomb, D. N., Ford, L. D., and Martin, R. W. Jr. (1990). Dressings and sauces. In Food Emulsions. Kare Larsson and Stig, E. Friberg (ed). p. 327 - 365. Marcell Deckker Inc. New York.

Houghten, R. A., DeGraw, S. T. (1987). Effect of positional and environmental domains on variation of HPLC peptide retention coefficient. J. Chromatogr. **386**, 223 - 228.

Huddleston, M. J., Bean, M. F., and Carr, S. A. (1993). Collisional fragmentation of glycopeptides by ESI LC/MS and LC/MS/MS: Methods for selective detection of glycopeptides in protein digests. Anal Chem. **65**: 877 - 884.

Hung, S. C., Zayas, J. F., (1991). Emulsifying Capacity and Emulsion Stability of Milk Proteins and Corn Germ protein Flour. J. Food Sci. **56**. 1216.

Ishida, J., Kai, M. and Ohkura, Y. (1986). High Performance Liquid Chromatography of Tyrosine Containing peptides by Precolumn Derivatization Involving Formylation Followed by Fluorescence Reaction with 1,2-Diamino-4,5-dimethoxy Benzene. J. Chromatogr. **356**: 171 - 177.

Jaffe, W. G., and Camejo, G. (1961). La accim de una protein a toxica aidadade caraotas negras (Phaseolus vulgaris) sorbre la absorcion intestinaql en ratas. Acta Cient Venez. **12**: 59. (Abstr).

Karas, M., Hillenkamp, P. (1988). Laser desorption ionization of proteins with molecular mass exceeding 10000 daltons. Anal Chem. **60**. 2299

Kato, A., and Nakai, S. (1980). Hydrophobicity determined by a fluorescence probe method and its correlation with surface properties of proteins. Biocim. Biophys. Acta. 624: 13 - 20.

Kato, A., Komatsu, K., Fjuimoto, K., and Kobayashi, K. (1985). Relationship between surface functional properties and flexibility of proteins detected by the protease susceptibility. J. Agric Food Chem. **33**: 931.

Katsumi, S., and Onodera, M. (1991). Effect of three kinds of dietary nitrogen sources on the metabolic fate of tryptophane. Agric Biological Chem. **55**: 2945.

Katsumi, S., Murotani, M., Onodera, M. (1992). Differences in Tryptophane-Nicotinamide Conversion According to Dietary Nitrogen Sources. Casein, Casein Hydrolysate, and Mixtures of Amino Acids. Biosci. Biotechnol. Biochem. **56**: (4). 665 - 669.

Kawasaki, H., Susuki, K (1990). Separation of Peptides Dissolved in SDS Solutions by Reversed Phase High Performance Liquid Chromatpgraphy: Removal of SDS from Peptides using an Ion Exchange Precolumn. Anal. Biochem. **186**, 264 - 268.

Kester, J. J., and Richardson, T. 1984. Modification of whey proteins to improve functionality. J. Dairy Sci. 67: 2757.

Khan, J. K., Kebede, N., Kuo, Y. H., Lambein, F., DeBruyn, A., (1993). Analysis of Neorotoxin β-ODAP and its α- isomer by Precolumn Derivatization with Phenylisothiocyanate. Anal. Biochem. **208**: 237 - 240.

Kim, Y. A., and Barbeau, W. E. (1991). Evaluation of SDS-page method for estimating protein digestibility. J. Food Sci. **56**: 1082.

Kinsella, J. E., (1976). Functional Properties of Proteins in Food: A survey. CRC Crit. Rev, Food Sci. Nutr. April. p 219 - 280.

Kinsella, J. E. (1981). Functional properties of proteins: Possible relationship between structure and functions in foams. Food Chem. **7**: 273.

Klemaszewski, J. L., Das, K. P., and Kinsella, J. E. (1992). Formation and coalescence stability of emulsions stabilized by different milk proteins. J. Food Sci. **57**: 366.

Kucera, P. Umagat, H. (1983). Design of a Post-Column Fluorescence Derivatization System for use with Microbore Columns. J. Chromatogr. **255**: 563 - 579.

Kumar, C. R., Hamed, A. (1991) Cummilative incidence of Atopic disorders in high risk infants fed whey hydrolysate, soy and conventional cow milk formula. Annals of Allergy. **67** (2 part 1) 129 - 132

Lemieux, L. and Amiot, J. (1989). Application of Reversed Phase high Performance Liquid Chromatography to the Separation of peptides from Phosphorylated and Dephosphorelated casein Hydrolysates. J Chromatogr. 473: 189 - 206.

Lemieux, L. puchardes, R. and Simard, R. E. 1989). Size Exclusion High Performance Liquid Chromatography Separation of Bitter and Astringent Fractions from Cheddar Cheese made with Added Lactobacillus Strain to Accelerate Ripening. J. Food Sci. **54**: 1235.

Li, Z., Alli, I., and Kermasha, S. (1989). Tryptic hydrolysis (in vitro) of crystalline and noncrystalline proteins from Phaseolus beans. J. Agric Food Chem. **37**: 643.

Li-Chan, E., Nakai, S., and Wood, D. F. (1984)Hydrophobicityand solubility of meat proteins and their relationship to emulsifying properties. J. Food Sci. **49**: 345.

Liener, I. E. (1976). Legume toxins in relation to protein digestibility: A review. J. Food Sci. **41**: 1076.

Liener, I. E., and Thompson, R. M. (1980). Invitro and invivo studies on the digestibility of the major storage protein of navy bean (Phaseolus vulgaris). Qual. Plant. Plant Foods. Hum Nutr. **30**: 13.

Little, E. L., Jeansonne, M. S., Foley, P. J. (1991). sequential Multimodal Elution for Pseudomultidimentional Liquid Chromatography on a Single Column. Anal. Chem. **63**, 33 - 44.

Loo, J. A., Udseth, H. R., Smith, R. D., (1989). Peptide and Protein Analysis by Eletrospray Ionization Mass Spectrometry and Capillary Electrophoresis Mass spectrometry. Anal. Biochem. **179**: 404 - 412.

Loo, J. A., Ogorzalek, L. R. R., Light, K. J., Edmonds, C. G., SmithR. D. (1992). Multiply charged negative ions by Electrospray ionization of polypeptides and proteins. Anal. Chem. **64,** 81 -88.

MacRitchie, F., (1985). Desorption of soluble components from mixed monolayers. J. Colloid Interface Sci. **107** (1). 276 - 7.

MacRitchie, F. (1978). Proteins at interfaces. Adv. Protein Chem. **32**: 283 - 324.

Mahmoud, M. I., Malone, W. T., and Cordle, C. T. (1992). Enzymatic hydrolysis of casein: Effect of degree of hydrolysis on antigenicity and physical properties. J. Food Sci. **57**: 1223 - 1229.

Mathews, D. M. (1991). Protein absorption: Development and present state of the subject. Wiley-Liss Inc., New York, NY.

Matsudaira, P. T., (1987). Sequence from Picomole Quantitie ot proteins Eletroblotted onto Polyvinylidene Difluoride Membrane. J. Biol Chem **262,** 10035.

Mazzeo, J. R., Martineau, J. A., and Krull, I. S. (1993). Peptide mapping using EOF-driven capillary isoelectric focusing. Anal. Biochem. **208**: 323 - 329.

Melnychyn, P. (1969). Process for isolating proteins from beans in a crystalline state using an alkaline salt of carboxylic acid. U.S. Patent 3,450,688.

Mendez, E., Matas, R., Soriano, F., (1985). Complete Automatization of Peptide Maps by Reversed Phase Liquid Chromatography Using Ortho-Phthalaldehyde Precolumn Derivatization. J. Chromatogr **323**: 373 - 382.

Meste, M. L., colas, B., Simatos, D., Closs, B., Courthaudon, J. I., and Lorient, D. (1990). Contribution of protein flexibility to the foaming properties of casein J. Food Sci. **55**: 1445.

Milce, M. J. C., Anguilar, M. I., Hearn, T. W. (1991). High Performance Liquid Chromatogrphy of Amino Acids, Peptides and Proteins. CVII[a] Analysis of Group Retention with a range of Mobile and Stationary Phases by Reversed Phase HPLC. J. Chromatogr. **536**: 165 -183.

Mitchell, J. R. (1986). Foaming and emulsifying properties of proteins. In Developments in Food Proteins. Vol. **4**. (B. J. F. Hudson, ed). Elsevier, London p. 291 - 338.

Mitchell, J. R., Irons, L., and Palmer, G. J. (1970). A study of the spread and adsorbed films of milk proteins. Biochim Biophys Acta, **200**: 138 - 150.

Mojarro-Guerra, S. H., Amado, R., Arrigoni, E., Solms, J. (1991). Isolation of Low Molecular Weight Taste Peptides from Vacherin Mont d'or Cheese. J. Food Sci. **56**, (4). 943.

Monjon, B. and Solms, S. J. (1987). Group Separation of peptides by Ligand Exchange Chromatography with a Sephadex Containing N-(2-Pyridylmethyl) glycine. Anal. Biochem. **160**: 88 - 97.

Montigny, P., Stobaugh, J. F., Givens, R. S., Carlson, R. G., Srinivasachar, K., Sternson, L. A., Higuchi, T., (1987). Naphthalene-2,3-dicarboxaldehyde / Cyanide Ion. A rationally designed Fluorogenic Reagent for primary Amines. Anal. Chem. **59**:1069 - 1107.

Moseley, M. A., Deterding, L. J., Tomer, K. B., (1991). Nanoscale Packed Capillary Liquid Chromatography with Mass Spectrometry Using Coaxial Continuous Flow Fast Atom Bombardment Interface. Anal Chem **63**: 1467 - 1473.

Nagarajan, G. R., Boone, S. J-L., stolzer, T. J. and Richieri, S. P. (1989). Effect of pH and ion pairing agents on the reversed phase high performance liquid chromatography of hydrophilic peptides: A case study of pentigetide and its fragments. "in Techniques in Protein Chemistry. (Ed. Tony, E. Hugli). academic Press Inc. pp. 357 - 362.

Nakai, S., and Li-Chan, E., (1988). Hydrophobic interactions in food systems. CRC Press Inc. Boca Raton Florida.

Nesheim, R. O. (1977). Food industry requirements for rapid methods of protein quality assessment. Food Technol. **31**: 87.

Nussbaum, M. A., Predwiecki, J. E., StaerK, D. U., Lunte, S. M., Riley, C. M. (1992). Electrochemical Characteristics of Amino Acids and Peptides Derived With Naphthalene-2,3-dicarboxyaldehyde: pH Effect and Differences in Oxidation Potentials. Anal Chem. **64**: 1259.

Nyberg, F., Pernow, C., Moberg, U., Eriksson, (1986). High Performance Liquid Chromatography and Diode Array Detection for the Identification of Peptides Containing Aromatic Amino Acids. In Studies of Endorphin Degrading Activity in Human Cerebrolspinal Fluid. J. Chromatogr. **352**: 359 - 368.

Oates, M. D., Jorgensen, J. W (1989). Determination of NAD-Labelled Amino Acids by Open Tubular Liquid Chromatography with Electrochemical Detection. Anal. Chem. **61**: 432 - 435.

Ochiai, K., Kamata, Y., Shibashaki, K. (1982). Effect of Tryptic Digestion on Emulsifying Properties of soy protein. Agric Biol Chem. **46** 91 - 96.

Ooi, T., Oobatake, M., Nemethy, G., and Scheraga, A. H. (1987). Accessing surface areas as a mearsure of the thermodynamic parameters of hydration of peptides. Proc. Natl. Acad. Sci. USA. **84** (7). 3086 - 3090.

Otani, H., Dong, X. Y., and Hosono, A. (1990). Preparation of low immunogenic peptide fragments from cow milk casein. Milchwisschaft. **45**: 217 - 220. (Abstr).

Pahud, J J. and Schwarz, K. (1984). Research and Development of Infant Formulae with reduced allergenic Properties. Ann. Allergy. **53**: 609 - 614.

Paredes-Lopez, O., and Harry, G. I., (1989). Changes in selectedchemical and antinutritional components during tempeh preparation using fresh and hardened common beans J. Food Sci. **54**: 968.

Paulson, A. T., and Tung, M. A. (1987). Solubility, hydrophobicity and net charge of succinylatedcanola protein isolate. J. Food Sci. **52**: 1557 - 1561.

Pearce, K. N., and Kinsella, J. E. 1978 Emulsifying properties of proteins: Evaluation of a turbidimetric technique. J Agric Food Chem. **26**: 716.

Phillips, R. D., Beuchat, L. R., (1981). Enzymatic Modification of Proteins, "In Protein Functionality in Foods" (J. P. Cheny. ed) ACS Symp. series **147** chap. 13.

Powrie, W. D., and Nakai, S., (1981) processing effects on protein systems: Utilization of protein resources (Stanley, D. W., Murray, E. D., and Lees, D. H., editors). Food and Nutr. Press: Westport Conn. 73 - 109.

Puski, G. 1975. Modification of functional properties of soya proteins by proteolytic enzyme treatment. Cereal Chem. **52**: 655 - 664.

Quaglia, G. B. abd Orban, E. (1990). Influence of enzymatic hydrolysis on structure and emulsifying properties of sardine (*Sardina pilchardus*) protein hydrolysates. J. food Sci. **55**: 1571 - 1573.

Rackis, J. J., Gumbmann, M. R., and Liener, L. E. (1985). The USDA trypsin inhibitor study. I. Background , objectives, and procedural details. Qual. Plant Foods Hum. nutr. **35**: 213 - 242.

Ravindranath, B., ed. (1990) Principles and Practice of Chromatography. Pub. Ellis Horwood Ltd. John Wiley and Sons. pp. 244.

Rebeca, B. D., Pena-Vera, M. T., Diaz-Castaneda, M. (1991). Production of Fish protein Hydrolysates with Bacterial Protease: yeild and Nutritional Value. J Food Sci. **56**:(2). 309

Rees, R. G., Raimundo, A. H., Grimble, G. K., Hunjan, M. K., Silk D. A. B. (1988). Peptide based Nitrogen source of enteral diet: Studies with Casein hydrolysates in man. J. of Parenteral and enteral Nutr. **12.** 215.

Rerat, A., Simons, N. C., Mendy, F., and Roger, L. (1988). Amino Acid absorption of pancreatic hormones in non- anaesthetized pigs after deodenal infusion of a milk enzymic hydrolysate or of free amino acids. Bri. J. of Nutr. **60,** 121- 136.

Sampson, H. A., Bernhisel, B. J., Yang, E., Scanlon, S. M. (1991). Safty of casein hydrolysate formular in children with cow milk allergy. J. of Pediatrics. **118.** (4 part 1). 520 -525.

Sathe , S. K., Ponte, J. G., Jr., Rangnekar, P. D., and Salunkhe, D. K. (1981). Effects of addition of the great northern bean (*Phaseolus vulgaris*) flour and protein concentrate on rheological properties of dough and baking qualityof bread. Cereal Chem., 58: 97

Sathe, S. K., Deshpande, S. S., and Salunkhe, D. K. (1984). Dry beans of Phaseolus. A review. Part 1. Chemical composition: Proteins. CRC Rev. Food Sci. Nutr., **20**(1)· 1.

Satterlee, L. D. (1981). Protein for use in foods. Food Technol. **35** (6), 53 - 70)

Savoie, L., and Gauthier, S. F. (1986). Dialysis cell for the in vitro measurement of protein digestibility. J. Food Sci. **51**: 494.

Schlabach, T. D. (1984). Postcolumn Detection of Serum proteins with the Biuret and Lowry Reaction. Anal. Biochem. **139**: 309 - 315.

Schlabach, T. D., Calburn, C. J., Maltaliano, J. R., Yuen, S. (1989). Meeting the challenge in peptide fragment purification for protein sequencing. "In Techniques in protein chemistry". (T. E. Hugli ed). Academic Press Inc. p. 497 -505.

Sessu, W. C., Rodgers, R. L., and Chichester, O. C. (1986). J. Chromatogr. **382**: 258 - 263.

Shimizu, M., Ametani, A., Kaminogawa, S., Yamauchi, K., (1986). The Topology of α-Casein adsorbed to an Oil/Water Interface: An Analytical Approach using Proteolysis. Biochemica et Biophysica Acta. **869**. 259 - 264.

Silk, D. B. A., Grimble, G. K., Rees, R. G. (1985). Protein Digestion and amino acid and Peptide Absorption. Proc. Nutr. Soc. **44**: 63.

Slighton, J. L., Sun, S. M., and Hall, T. C. (1983). Complete nucleotide sequence of a French bean storage protein gene: Phaseolin Proc. Natl. Acad. Sci. USA. **80**: 1897 - 1901.

Soble, H. A. (1989). Characterization of Peptides: "In A Practical Guide to Protein and Peptide Purification for Microsequencing". (Matsudaira P. T. ed). Academic Press Inc. San Diego. p 89 -109.

Song, B. K., Damodaran, S. (1987). Structure, Functional Relationship of Proteins: Adsorption at Air/Water Interface. J Agric Biol. Chem. **35**. 236.

Stanley, D. W., and Aguilera, J. M. (1985). A Review of textural defects in cooked reconstituted legumes. the influence of storage and processing. J. Food Biochem. **9**: 277.

Steel, R. G. D., and Torrie, J. H., (1980). Principles and Procedures of Statistics. A biometrical approach (2nd Edition). Chap. 17, page 411.

Stone, K. L., LoPresti, M. B., Williams, N D., Crawford, M. J., DeAngelis, R , Williams, K. R. (1989) Enzymatic digestion of proteins and HPLC peptides in the sub nanomole range. "In Techniques in protein Chemistry." (T. E. Hugli. ed) Academic press Inc. Harcourt Bruce Jovanovich. p 377 - 391.

Stone, K. L., Williams, K. R. (1986). High Performance liquid Chromatographic peptide mapping and amino acid analysis in the sub nanomole range. J. Chromatogr. **359**. 203 -212

Stone, K. L., Mary, B. L., Myron, J. C., Raymond, D., Kenneth, R. W. (1989). Enzymatic digestion of protein and HPLC peptide isolation. "In A practical Guide to protein and peptide purification for microsequencing". (P. T. matsudaira. ed). Academic Press Inc. San Diego. p 33 -47.

Sun, Y., Smith, D. L., Shoup, R. E., (1991). Simultaneous Detection of thiol and disulfide Containing Peptides by Electrochemical High Performance Liquid Chromatography with Identification by Mass Spectrometry. Anal Biochem. **197**: 69 - 76.

Swadesh, J. K. (1990). Tryptic Fingerprinting on Poly(styrene, Divinyl benzene) Reversed Phase High Performance Liquid Chromatographic Columns. J. Chromatogr. **512**, 315 - 323.

Tapuhi, Y., Schmdt, D. E., Lindner, W., Karger, B. L., (1981). Dansylation In Amino Acids for High Performance Liquid Chromatographic Analysis. Anal. Biochem. **115**: 123 - 129.

Taylor, W. H. (1957). Formal titration: An evaluation of its various modifications. The Analyst. **82**, 488 -497.

Tornberg, E., Ediriweera, N. (1987). Coalescence Stability of protein Stabilized Emulsions. "In Food Emulsions and Foams". (Ed. Dickinson. E.). Royal Soc. Chem. 55: 52.

Tornberg, E., Olsson, A.,and Persson, K. (1990). The structural and interfacial properties of food proteins in relation to their functions in emulsions. In Food Emulsions. Kare Larsson and Stig, E. Friberg (ed). p. 247. Marcel Dekker Inc. New York.

Townsend, A. A., and Nakai, S.)1983). Relationship between hydrophobicity and foaming characteristics of food proteins. J Food Sci. **48**: 588 - 594.

Trevor, P. (1991). Understanding Enzymes: Third Edition. (Ellis Harwood. NTD. p 87 - 88

Tsai, H. and Weber, S. G. (1990). Electrochemical Detection of Dipeptides and dipeptide Amides. J. Chromatogr. **515**: 451 - 457.

Tsai, H. and Weber, S. G. (1991). Electrochemical Detection of Oligopeptides through Precolumn Formation of Biuret Complex.

Turgeon, S. L., Gauthier, S. F., and Paquin, P. 1992. Emulsifying properties of whey peptide fractions as a function of pH and ionic strength. J Food Sci **57**: 601 - 604.

Turgeon, L. S., Gauthier, S. F., and Paquin, P. (1991). Interfacial and emulsifying properties of whey peptide fractions obtained with a two step ultrafiltration process. J. Agric Food Chem. **39**: 673 - 676.

van Breemen, R. B., Davis, R. G., (1992) Rate of peptide proteolysis measured using liquid chromatography and continuous flow fast atom bombardment mass spectrometry. Anal Chem. **64**: 2233 -2237.

Van-Leeuweu, H. J. (1978). Removal of bitter peptides from tryptic hydrolysates of casein coprecipitate by immunoabsorbent affinity chromatography. Agric Biol. Chem. **42**, 1375 -1378.

Warner, A. M. and Weber, S. G. (1991). Electrochemical detection of Peptides . Anal Chem. **61**: 2664 - 2668.

Webb, K. E., Matthews, J. C., and DiRienzo. D. B. (1992). Peptide absorption: A review of current concepts and future perspectives. J. Anim. Sci. **70**(10): 3248.

Webb, K. E. Jr., Dirienzo, D. B., Mathews, J. C. (1993). Recent developments in gastrointestinal absortption and tissue utilization of Peptides: A Review. J. Dairy Sci. **76**: 351 - 361.

Webb, K. E. Jr. (1990). Intestinal Absorption of Protein Hydrolysis Products: A Review. J. Anim. Sci. **68**: 3011.

Welch, R. W.., Acworth, F , and Leuine, M. (1993). Coulometric electrochemical detection of hydroxyproline using 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. Anal. Biochem. 210: 199 - 203.

Whitaker, J. R and Puigserver, A. J. (1982). "In Modification of Proteins: Food Science and Pharmacological Aspects" (Ed. R. E. Feeney and J. R. Whitaker) Advances in Chemistry Series. 198. Am. Chem. Soc. Washington D.C. USA. pp. 57 - 87.

Willoughby, K. C., and Browner, R. F., (1984). Monodispersed Aerosol Generation Interface for Combining Liquid Chromatography with Mass Spectroscopy. Anal Chem. **56**: 2626.

Winarno, F. G. (1979). Fermented vegetable protein and related foods of South East Asia with Spercial reference to Indonesia. J. Am. Oil Chemist Soc. **56.** 363 -366.

Yamashita, M., Arai, S., Matsuyamu, J., Gonda, M., Kato, H., Fujimaki, M. (1970) Enzymatic modification of proteins in foodstuff. Agric Biol Chem. **34,** 1484 - 1491.

Pomeranz, Y. 1991. Functional properties of food components 2nd Edition. Academic Press, Inc. Chap. (6). p. 238)

Yoshida, T., Uetake, A., Murayama, H., Nimura, N. and Kinoshita, T. (1985). Fluorescent Labelling of Amino Acids with 9-Aanthryldiazomethane and its Application to High Performance Liquid Chromatogr **348**: 425 - 429.

Young, M. P., Wheat, T. E. (1990). Optimization of High Performance liquid chromatography peptide separation with alternative mobile and stationary phases.

Young, M. P., Merion, M. (1989). Capillary Electrophoresis analysis of species variation in the tryptic maps of cytochrom C. "In Currenr Research in protein chemistry· Techniques, Structure, and Function." (J J. Villafraca. ed) Academic Press Inc. p 217 -232.

APPENDIX A

Table A1.

Analysis of Variance for NSI

Source	DF	Sum of Squares	Mean Square	F Value
Model	8	3501 821647	437.727706	27.33***
Error	23	368.346441	16.015063	
Total	31	3870.168088		
LNT	1	354.563959	354.563959	22.14***
BEAN	3	303.797462	101.265821	6.32***
TRT	1	2437.067113	2437.067113	152.17***
BEAN*TRT	3	406.393113	135.464371	8.46***

*** - Significant at the 0.1% probability level.
R squared = 0.905.

Table A2.

BEAN	NSI LSMEAN	Std Err
BLB	13.9662500	1.4148791
LLB	11.0587500	1.4148791
NB	10.6700000	1.4148791
WKB	18.3775000	1.4148791

Table A3.

TRT	NSI LSMEAN	Std Err
CRYS	22.2450000	1.0004706
AMOR	4.7912500	1.0004706

Table A4.

BEAN	TRT	NSI LSMEAN	Std Err
BLB	CRYS	22.1350000	2.0009412
LLB	AMOR	5.7975000	2.0009412
LLB	CRYS	17.5375000	2.0009412
LLB	AMOR	4.5800000	2.0009412
NB	CRYS	16.2450000	2.0009412
NB	AMOR	5.0950000	2.0009412
WKB	CRYS	33.0625000	2.0009412
WKB	AMOR	3.6925000	2.0009412

Table A5.

Parameter	Estimate	T-test	Std Err
WKB/CRYS VS WKB/AMOR	29.37000000	10.38***	2.82975817
NB/CRYS VS NB/AMOR	11.15000000	3.94***	2.82975817
BLB/CRYS VS BLB/AMOR	16.33750000	5.77***	2.82975817
LLB/CRYS VS LLB/AMOR	12.95750000	4.58***	2.82975817
CRYS VS AMOR	17.45375000	12.34***	1.41487909

*** - Significant at the 0.1% probability level.

Appendix B:

Table B1.

Analysis of Variance for DH

Source	DF	Sum of Squares	Mean Square	F Value
Model	8	1.45384041	0.18173005	10253***
Error	39	0.06912640	0.00177247	
Total	47	1.52296681		
LNT	1	1.07288143	1.07288143	60530***
BEAN	3	0.20331573	0.06777191	38.24***
TRT	1	0.01508752	0.01508752	8.51ns
BEAN*TRT	3	0.16255573	0.05418524	30.57***

*** -Significant at the 0.1% probabolity level.
ns -non significant at the 0.1% probability level.
R squared =0.955.

Table B2.

BEAN	DH LSMEAN	Std Err
BLB	0.42925000	0.01215344
LLB	0.43308333	0.01215344
NB	0.46116667	0.01215344
WKB	0.58875000	0.01215344

Table B3.

TRT	DH LSMEAN	Std Err
CRYS	0.49579167	0.00859378
AMOR	0.46033333	0.00859378

Table B4.

BEAN	TRT	DH LSMEAN	Std Err
BLB	CRYS	0.36383333	0.01718755
BLB	AMOR	0.49466667	0.01718755
LLB	CRYS	0.44733333	0.01718755
LLB	AMOR	0.41883333	0.01718755
NB	CRYS	0.48433333	0.01718755
NB	AMOR	0.43800000	0.01718755
WKB	CRYS	0.68766667	0.01718755
WKB	AMOR	0.48983333	0.01718755

Table B5.

Parameter	Estimate	T-Test	Std Err
WKB/CRYS VS WKB/AMOR	0.19783333	8.14***	0.02430687
NB/CRYS VS NB/AMOR	0.04633333	1.91ns	0.02430687
BLB/CRYS VS BLB/AMOR	-0.13083333	-5.38***	0.02430687
LLB/CRYS VS LLB/AMOR	0.02850000	1.17ns	0.02430687
CRYS VS AMOR	0.03545833	2.92ns	0.01215344

*** - Significant at the 0.1% probability level.
ns -Non significant at the 0.1% probability level.

Amino acid	Letter codes	Molecular formula	Monoiso.MW[a] in free state	Aver.MW[b] in free state	Monoiso. mass in peptide chain[c]	Aver. mass in peptide chain[c]
Alanine	Ala (A)	$C_3H_7NO_2$	89.0477	89.0938	71.0371	71.0786
Arginine	Arg (R)	$C_6H_{14}N_4O_2$	174.1117	174.2022	156.1011	156.1870
Asparagine	Asn (N)	$C_4H_8N_2O_3$	132.0535	132.1188	114.0429	114.1036
Aspartic acid	Asp (D)	$C_4H_7NO_4$	133.0375	133.1036	115.0269	115.0884
Asn and/or Asp	Asx (B)	—	—	—	—	—
Cysteine	Cys (C)	$C_3H_7NO_2S$	121.0197	121.1538	103.0091	103.1386
Glutamine	Gln (Q)	$C_5H_{10}N_2O_3$	146.0691	146.1456	128.0585	128.1304
Glutamic acid	Glu (E)	$C_5H_9NO_4$	147.0532	147.1304	129.0426	129.1152
Gln and/or Glu	Glx (Z)	—	—	—	—	—
Glycine	Gly (G)	$C_2H_5NO_2$	75.0320	75.0670	57.0214	57.0518
Histidine	His (H)	$C_6H_9N_3O_2$	155.0695	155.1560	137.0589	137.1408
Isoleucine	Ile (I)	$C_6H_{13}NO_2$	131.0946	131.1742	113.0840	113.1590
Leucine	Leu (L)	$C_6H_{13}NO_2$	131.0946	131.1742	113.0840	113.1590
Lysine	Lys (K)	$C_6H_{14}N_2O_2$	146.1055	146.1888	128.0949	128.1736
Methionine	Met (M)	$C_5H_{11}NO_2S$	149.0510	149.2074	131.0404	131.1922
Phenylalanine	Phe (F)	$C_9H_{11}NO_2$	165.0790	165.1914	147.0684	147.1762
Proline	Pro (P)	$C_5H_9NO_2$	115.0633	115.1316	97.0527	97.1164
Serine	Ser (S)	$C_3H_7NO_3$	105.0426	105.0932	87.0320	87.0780
Threonine	Thr (T)	$C_4H_9NO_3$	119.0582	119.1200	101.0476	101.1048
Tryptophan	Trp (W)	$C_{11}H_{12}N_2O_2$	204.0899	204.2280	186.0793	186.2128
Tyrosine	Tyr (Y)	$C_9H_{11}NO_3$	181.0739	181.1908	163.0633	163.1756
Valine	Val (V)	$C_5H_{11}NO_2$	117.0790	117.1474	99.0684	99.1322

[a] The monoisotopic MW is calculated from the most abundant isotopes of the elements in the molecular formula.
[b] The average MW is the abundance-weighted average of all elemental masses in the molecular formula.
[c] To link N amino acids together to form a peptide chain, N-1H_2O molecules have to be removed. Therefore, the mass of each amino acid residue in a peptide chain is the MW of the free amino acid less the MW of H_2O (18.0106 for the monoisotopic and 18.0152 for the average). The monoisotopic and average MW of a protein can thus be simply calculated by summing up the corresponding masses of all residues in the protein sequence and then adding the corresponding MW of one H_2O; the mass of hydrogen has to be subtracted for each cysteine that forms disulfide bond.

Feng et. al., (1991).