Enzymatic Hydrolysis and Peptide Mapping of Potato Pulp Protein

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Erklärung

Ich versichere, dass ich diese Dissertation selbstständig und nur unter Verwendung der angegebenen Hilfsmittel und Quellen durchgeführt habe. Diese Arbeit wurde nicht als Diplomarbeit oder ähnliche Prüfungsarbeit verwendet.

Chulaporn Kamnerdpetch

Hannover, den 29. Mai 2006

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ABSTRACT

The enzymatic hydrolysis of potato pulp (PP) was carried out by using four different enzymes, i.e. Alcalase (ALC), Novo Pro-D (NPD), Flavourzyme (FLA), and Corolase (COR), and eight combination of proteolytic enzyme systems in a 4 l batch reactor at 50 °C for 26 h without pH control. The endoprotease ALC and exopeptidase FLA are most suitable for hydrolyzing the PP protein. The combination of an endoprotease, ALC or NPD, and an exopeptidase, FLA, is more appropriate than using the individual proteases. The amount of FLA influences the degree of hydrolysis (DH) and the amount of the obtained end products. The highest DH, 44 %, and total amount of free amino acids (Σ faa), 306 mg/g protein, were obtained from the combination of 2 % ALC + 5 % FLA (w/w). The amino acid concentration in the enzymatic hydrolysates is significantly higher than in the native potato pulp (NPP), especially aromatic amino acids (His, Phe, Trp, and Tyr) and the sulfur-containing amino acid methionine (Met).

The effects of the influences of various experimental parameters on the RP-HPLC separation were examined. (i) Chromatographic results from four different alkyl chain lengths (C_4, C_8, C_{12}, and C_{18}) as well as from the matrix´ different particle and pore sizes were comparable. (ii) A chromatographic condition such as an effect of the hydrophobic strength of organic solvents in the mobile phase and influence of the temperature on the peptide retention and the selectivity have been studied. The alkyl chain length and particle and pore size of the stationary phase affected the separation efficiency (resolution) but did not affect the selectivity. The resolutions of hydrophilic peptides eluted from C_8 and C_{18} columns and the hydrophobic peptides eluted from C_{12} column were improved when eluted with acetonitrile (ACN) gradient and iso-propanol (IPA) gradient, respectively. The retention time and peak width decreased as temperature increased from 30 to 50 °C. Increasing temperature resulted in the co-elution of hydrophobic peptides when eluted with ACN and IPA.

The PP hydrolysates peptides were separated by size exclusion chromatography (SEC) on a Superdex Peptide HR 10/30 column. Influencing factors, e.g. injection volume and various mobile phase types, for the peak resolution were investigated. A non-ideal size exclusion effect was observed for some charged amino acids and peptides containing hydrophobic residues. The PP hydrolysates were separated into seven fractions (S1 to S7) by using a low injection volume (20 µl) and eluted with 20 % ACN + 0.1 % TFA. No contamination of amino acids occur in the fractions S1 and S2, while fractions S3 to S7 were contaminated, especially Trp in fraction S7. SEC fractions (S1 to S7) were further separated

on RP-HPLC with σ-phthalaldehyde (OPA) derivatization and non-derivatization methods. The separation signal of low molecular weight fractions (S3 to S7) by the OPA derivatization method was superior to the non-derivatization method. The superior selectivity and the shorter retention time were realized from the non-derivatization method by using a ACN gradient. Satisfactory separation of fraction S6 and S7 were detected. The S7 fraction mainly contains small hydrophobic peptides.

The masses of the peptides in the SE-RP separated fractions were characterized by MALDI and LC/ESI-MS on the positive mode. MALDI analysis of the SE-RP chromatograph fractions cannot provide an accurate molecular mass distributions of the peptides. The doubly charged $[M + 2H]^{2+}$ species were observed by LC/ESI-MS. The S7R4 and S7R8 fractions contain the same molecular mass but different hydrophobicity peptides. Moreover, the one mass unit difference between the ions was detected in the LC/ESI-MS spectra. This difference resulted out of (i) the replacement of a ^{12}C atom by a ^{13}C atom, or (ii) two peptides having identical residues but a one single mass unit difference of their internal amino acid pairs. The cysteine or methionine oxidation $[M + O]^{+}$ ion and the sodium adduct $[M + Na]^{+}$ ion were also recognized.

Key words

Potato pulp, enzymatic hydrolysis, endoprotease, exopeptidase, degree of hydrolysis, amino acid, peptide, RP-HPLC, SEC, MALDI-MS, ESI-MS

KURZFASSUNG

In einem 4 l Batch-Reaktor ohne pH-Kontrolle wurde die enzymatische Hydrolyse von Kartoffelkleber (Potato pulp, PP) für 26 h bei 50 °C durchgeführt. Hierzu wurden die Enzyme Alcalase (ALC), Novo Pro-D (NPD), Flavourzyme (FLA) und Corolase (COR) sowohl einzeln als auch in acht unterschiedlichen Kombinationen untersucht. Die Endoprotease ALC und Exopeptidase FLA sind zur Hydrolyse des PP-Proteins am besten geeignet. Die Kombination einer Endoprotease (ALC oder NPD) mit einer Exopeptidase (FLA) ist geeigneter als die Verwendung der einzelnen Proteasen. Die Menge von FLA beeinflusst den Hydrolysierungsgrad (DH) und die Menge der erhaltenen Endprodukte. Der höchste DH (44 %) und die maximale Gesamtmenge an freien Aminosäuren (Σ faa; 306 mg/g Protein) wurden durch eine Kombination von 2 % ALC und 5 % FLA (w/w) erreicht. Die Aminosäurekonzentration der enzymatischen Hydrolysate ist deutlich höher als in dem nativen Kartoffelkleber (NPP); dies trifft insbesondere auf die aromatischen Aminosäuren (His, Phe, Trp und Tyr) und die schwefelhaltige Aminosäure Methionin (Met) zu.

Zudem wurde der Einfluss verschiedener Parameter auf die RP-HPLC-Trennung untersucht. (i) Dies führte zur Vergleichbarkeit der chromatographischen Ergebnisse von vier verschiedenen stationären Phasen mit unterschiedlichen Alkyl Ketten-Längen (C_4, C_8, C_{12} und C_{18}) sowie von unterschiedlichen Partikel- und Porengrößen der Matrix. (ii) Bei der Optimierung der chromatographischen Bedingungen wurden sowohl der Effekt der Stärke der Hydrophobizität der organischen Lösemittel in der mobilen Phase als auch der Einfluss der Temperatur auf die Peptid-Retention und die Selektivität untersucht. Es stellte sich heraus, dass die Länge der Alkylkette sowie die Partikel- und Porengröße der stationären Phase zwar einen Einfluss auf die Effizienz der Auftrennung (Auflösung) haben, aber nicht auf die Selektivität. Die Auflösung der hydrophilen Peptide, welche von C_8- und C_{18}-Säulen, bzw. der hydrophoben Peptide, welche von der C_{12}-Säule eluiert wurden, konnte durch Verwendung eines Acetonitril- (ACN) bzw. Isopropanol- (IPA) Gradienten verbessert werden. Dies spiegelte sich wider in kürzeren Retentionszeiten und Peak-Breiten bei einer Temperaturerhöhung von 30 auf 50 °C. Eine Steigerung der Temperatur ergab eine Coelution von hydrophoben Peptiden beim Eluieren mit ACN und IPA.

Die PP-Hydolysat-Peptide wurden mithilfe der Größenausschluss-Chromatographie (SEC) auf einer Superdex Peptide HR 10/30-Säule getrennt. Hierbei wurden Faktoren wie Injektionsvolumen und verschiedene Typen der mobilen Phase, die einen Einfluss auf die Peak-Auflösung haben, untersucht. Ein nicht-idealer Größenausschlusseffekt wurde beobachtet

bei einigen geladenen Aminosäuren und Peptiden, welche hydrophobe Reste enthalten. Die PP-Hydrolysate wurden in sieben Fraktionen aufgeteilt (S1 bis S7) durch Verwendung eines geringen Injektionsvolumens (20 µl) und mit 20 % ACN und 0,1 % TFA eluiert. Die ersten beiden Fraktionen waren dabei frei von Aminosäuren; Fraktionen S3 bis S7 waren kontaminiert (speziell mit Trp in Fraktion S7). Die so erhaltenen SEC-Fraktionen wurden anschließend mit einer RP-HPLC aufgetrennt; teilweise wurde im Anschluss eine Derivatisierung mit σ-Phthalanhydrid (OPA) durchgeführt. Das Auftrennungs-Signal der Fraktionen mit niedrigerem Molekulargewicht (S3 bis S7) war nach der OPA-Derivatisierung deutlich höher als bei der Methode, in welcher dieser abschließende Schritt fehlte. Höhere Selektivität und kürzere Retentionszeiten konnten allerdings durch Verwendung eines ACN-Gradienten und ohne Derivatiserung realisiert werden. Hierdurch konnte eine befriedigende Trennung der Fraktionen S6 und S7 erreicht werden, wobei die letzte Fraktion ausschließlich kleine hydrophobe Peptide enthielt.

Eine Bestimmung der molaren Massen der Peptide in den Fraktionen, welche mithilfe der SE-RP aufgetrennt wurden, erfolgte anschließend mit MALDI und LC/ESI-MS im positiven Modus. Es zeigte sich, dass die MALDI-Analyse der SE-RP-Fraktionen keine genaue Verteilung der molaren Massen liefern kann. Doppelt geladene $[M+2H]^{2+}$-Spezies wurden mit LC/ESI-MS beobachtet. Die Fraktionen S7R4 und S7R8 enthielten Peptide gleicher molarer Masse mit unterschiedlicher Hydrophobie. Zudem wurden in den LC/ESI-MS-Spektren Ionen detektiert, welche einen Massen-Unterschied von 1 aufwiesen. Hierbei handelte es sich entweder um Moleküle, (i) in denen ein ^{12}C-Atom durch ein ^{13}C-Atom ausgetauscht wurde, oder (ii) um zwei Peptide mit einem Massen-Unterschied von 1 zwischen ihren jeweiligen Aminosäurenpaaren. Zudem wurden sowohl die oxidierten Cystein- und Methionin-Ionen $[M + O]^+$ als auch die entsprechenden Natrium-Addukte $[M + Na]^+$ detektiert.

Schlüsselwörter

Kartoffelkleber, enzymatische Hydrolyse, Endoprotease, Exopeptidase,
Hydrolysierungsgrad, Aminosäure, Peptid, RP-HPLC, SEC, MALDI-MS, ESI-MS

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ABBREVIATIONS

$(M + H)^+$	singly charged ion
$(M + nH)^{n+}$	multiply charged ion
$[M + 2H]^{2+}$	doubly charged ion
$[M + Na]^+$	adduction of sodium ion
$[M + O]^+$	oxidated ion
A	acceptor
ACN	acetonitrile
ALC	Alcalase
Arb	arbitrary units
Arg	arginine
Asn	asparagine
Asp	aspartate
Asx	asparagine and aspartate
C_4	n-butyl
C_8	n-octyl
C_{12}	n-dodecyl
C_{18}	n-octadecyl
COR	Corolase
Cys	cysteine
D	donor
Da	dalton
DH	degree of hydrolysis
DHB	2,5-dihydroxy-benzoic acid
E	enzyme
EC	Enzyme Commission
ESI	electrospray ionization
FAB	fast atom bombardment
FLA	Flavourzyme
GFC	gel filtration chromatography
Gln	glutamine
Glu	glutamate
Glx	glutamine and glutamate
h_{tot}	total number of peptide bonds presented in the parental protein
h	number of peptide bonds hydrolyzed
H^+	proton
His	histidine
IPA	isopropanol
K	reaction velocity constants

k	retention factor
K^+	potassium ion
LC	liquid chromatography
LC/MS	liquid chromatography and mass spectrometry
Leu	leucine
Lys	lysine
m/z	mass-to-charge ratio
MALDI	matrix-assisted laser desorption/ionization
Met	methionine
MS	mass spectrometry
MW	molecular weight
NC-IUBMB	The Nomenclature Committee of the International Union of Biochemistry and Molecular Biology
NH_4^+	ammonium ion
NPD	Novo Pro-D
NPP	native potato pulp
OPA	σ-phthalaldehyde
P	peak capacity
P and P´	resulting peptides
PBS	0.25 M NaCl in 0.02 M phosphate buffer pH 7.2
Phe	phenylalanine
PP	potato pulp
PSD	post source decay
RPC	reverse-phase chromatography
RP-HPLC	reverse-phase high performance liquid chromatography
S	substrate
SA	3,5-dimethoxy-4-hydroxy-cinnamic acid
SEC	size exclusion chromatography
t	tons
TFA	trifluoroacetic acid
Th	Thomson
THF	tetrahydrofuran
TNBS	trinitrobenzenesulphonic acid
TOF	time-of-flight
Trp	tryptophan
Tyr	tyrosine
z	charge
Zn	zinc
α–cyano	α–cyano-4-hydroxy-cinnamic acid
Σ faa	total amount of free amino acid

CHAPTER 1 INTRODUCTION

Food wastes such as agricultural wastes and food processing by-products, represent a potential source of energy and are valuable materials. Several tons of agro-industrial by-products are produced each day which vary in the chemical composition. Their chemical properties are composed of protein concentration and its biological value, quantitative and qualitative composition of amino acids, digestibility level of energy fats and carbohydrates, vitamin and mineral content, and the amount of fiber and substances.

Potato starch was first produced in Germany since the end of seventeenth century. The process is relatively simple and yields 160-180 kg of starch per ton of potatoes (Swinkels, 1990). It can be used for many purposes, i.e. in the paper and textile industry, food industry, and for the production of glucose. By producing potato starch, a large amount of low valuable by-products, with regard to both potato fruit water and potato pulp, is produced. Potato pulp (PP) remains after grinding the potato and washing out the starch. In Table 1.1 the compositions of a typical wet sample and untreated potato pulp are listed. On a dry matter basis the pulp contains 74 % of protein which corresponds to 1.5-2.5 % of protein in the potato tuber. This contains a higher proportion of the essential amino acid lysine than most cereal proteins, but is deficient in the sulphur-containing amino acids, methionine and cysteine (Ralet, 1999; Kapoor, 1975; and Kanorr, 1978). Therefore it can be used mainly for animal feed.

Table 1.1 Composition of conventional wet potato pulp (Mayer, 1997)

Component	**Relative to wet pulp (% w/w)**	**Relative to dry matter (% w/w)**
Dry matter	13.0	-
Total organic matter	12.5	96
Ashes	0.5	4
Starch	4.9	37
Cellulose	2.2	17
Hemicellulose	1.8	14
Pectin	2.2	17
Fiber (unidentified)	0.9	7
Protein / amino acids	0.5	4

A more profitable utilization of potato proteins by-products would be of economic interest for potato starch factories (Van Koningsveld, 2001; Kanorr, 1982; Kanorr, 1977). Thus, several attempts have been made to receive more valuable and useful products. Enzymatic hydrolysis is one of the main methods for altering food proteins (Hrčková, 2000; Franek, 2000). There are many commercially available enzymes that may be used. Proteases are very commonly used to partially hydrolyse proteins under mild conditions with very high and reproducible specificity (Rao, 1998; Adler-Nissen, 1993). During an enzymatic hydrolyzation process proteins are cleaved to smaller molecules, i.e. smaller peptides and free amino acids. Hence, the nutrition quality and safety of the products improves. New products could be generated and alternative applications for several agricultural products realized.

Peptide mapping is a powerful technique widely used by many protein chemistry researchers to examine the obtained peptides. A general approach to peptide mapping is the protein hydrolysis followed by separation and characterization of the obtained peptides which results in multidimensional maps. Lots of chromatography techniques are used to separate peptide mixtures into its constituting fractions for mapping. The successful techniques are shown in Table 1.2. Utilization of any of these methods usually results in increasing the purity of the interested peptide and a highly purified sample yield may be acquired by a combination used of these methods. The separation bases on the differences of the sample's affinity towards the mobile and the stationary phase.

Normally, a peptide mixture, that results from a digestion of the protein, is fractionated by one or two steps of a liquid chromatography. Size exclusion chromatography (SEC) and reverse-phase high performance liquid chromatography (RP-HPLC) are attractive procedures for investigating peptide profiles in protein hydrolysates (Visser, 1992; Pellerin, 1985). RP-HPLC predominates and is often the method of choice for peptide separation (Jaroniec, 1993; Hearn, 1991). This method is able to separate the molecules that have some degree of hydrophobic character, such as proteins and peptides, with excellent recovery and resolution (Krstulovic, 1982), while SEC or gel filtration chromatography (GFC) is a widely used and well documented method to analyze the molecular weight of peptides from protein hydrolysates. It continues to be an efficient separation method in peptide purification and presents the most simple separation mechanism in chromatography. Knowing the molecular weight distribution of peptides in the protein hydrolysates is essential for predicting the end-use characteristics of the product and for quality control.

Table 1.2 Chromatographic techniques most commonly used in peptide and protein purification protocols with their basis of separation (Walsh, 2002)

Techniques	**Basis of separation**
Ion exchange chromatography	Differences in protein surface charge at a given pH
Gel filtration chromatography	Differences in mass or shape of different proteins
Affinity chromatography	Based upon biospecific interaction between a protein and an appropriate ligand
Hydrophobic interaction chromatography	Differences in surface hydrophobicity of proteins
Reversed-phase chromatography	Differences in surface hydrophobicity of proteins
Chromatofocussing	Separates proteins on the basis of their isoelectric points
Hydroxyapatite chromatography	Complex interactions between proteins and the calcium phosphate-based media. Not fully understood.

The peptide fragments from the initial separation steps need to be characterized by different approaches such as molecular mass, amino acid composition, and sequencing, which cannot be achieved by RP-HPLC or SEC. The different molecules have different masses so the molecules present in the sample can be determined by mass spectrometry. This technique is the most recent and sensitive analytical technique for the determination of peptides and proteins molecular mass. It allows not only the precise determination of the peptides or proteins molecular mass, but also the determination of their sequences (Burlingame et al., 1996; Eckart, 1994; and McCloskey, 1990). At the beginning of the 1990s two new ionization methods were developed: (i) electrospray ionization (ESI) (Fenn et al., 1989), and (ii) matrix-assisted laser desorption ionization (MALDI) coupled to time-of-flight (TOF) analyzers (Karas and Hillenkamp, 1988). These are now routine to analyze peptides and proteins at femtomole levels which make them well suited for the mass mapping and structural determination (Lewis et al., 2000).

Since not much is known about the effective process for modifying and utilizing potato pulp proteins, the aim of this study was to find a proper enzyme combination for the production of PP protein hydrolysates with a high degree of hydrolysis and a quality improvement. PP was enzymatically hydrolyzed with four individual enzymes and eight different enzyme combination systems. The degree of hydrolysis and the distribution of amino acids in the hydrolysates were investigated. The results of this study can provide a more effective and precise method for protein hydrolysis of potato pulp.

Furthermore, the development of various chromatographic method for the separation of peptides from PP hydrolysates to obtain the chromatographic purity of the peptide peaks were done. For this purpose, SEC method was used to separate the peptides in PP hydrolysates into different molecular size fractions by a Superdex Peptide HR10/30 column with various mobile phases. Each fraction obtained from SEC was further separated by RP-HPLC.

The main resulting peptide fractions from RP-HPLC were further characterized by Matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) mass spectrometry. Hence, a characteristic information of each peptide fraction obtained from these investigation deliver not only information about the process but also information to predict the end-use of the products.

CHAPTER 2 THEORY

2.1 Potato starch production

The increasing of human population in Europe over the last decades has greatly influenced the demand for food products. Potato (*Solanum tuberosum*) is a major world crop product of which 300 million tons are produced worldwide annually (FAO, 2000). It is the most important vegetable in European countries today. Potatoes are used for several purposes, including human consumption, industrial processing (potato starch, alcohol etc.), and recultivation (Feustel, 1987; Talburt, 1987). Fresh potato contains about 17-21 % starch, 0.5-1.2 % of proteins, mineral compounds, organic acids, and ash (Liu, 2002; Smith, 1977). Therefore, it can be used as a raw material for starch production. Table 2.1 compares a starch production by raw material in the EU, the US, and other countries. In the case of wheat and potato starch, the EU community is the leader in these sectors.

Table 2.1 Starch output from raw material in the EU, US, and other countries, 2000 (million tons, native starch)

Region	**Maize**	**Potatoes**	**Wheat**	**Other**	**Total**
EU	3.9	**1.8**	2.8	0.0	8.4
US	24.6	0.0	0.3	0.0	24.9
Other Countries	10.9	0.8	1.1	2.5	15.2
World	39.4	2.6	4.1	2.5	48.5

Source: European Commission (DG Agriculture, Unit C2), United States Department of Agriculture and LMC estimates.

In 2000 European Union consisted of 15 member states. Thus, the EU industry is subject to production quotas. Only 8 of 15 member countries, i.e. Austria, Germany, Denmark, Finland, France, the Netherlands, Spain, and Sweden, have an EU quota for potato starch. The maximum potato starch production in the years 2001 and 2002 has been fixed at approximately 1,762 X 10^3 tons (t). The main share is covered by four member states: Germany (656 X 10^3 t), the Netherlands (507 X 10^3 t), France (265 X 10^3 t), and Denmark (168 X 10^3 t). Smaller quotas 48, 53, and 62 X 10^3 t are held by Austria, Finland, and Sweden, respectively, while Spain owns a quota of 2000 t (Fig. 2.1) (Sohn, 1997; LMC International, 2000).

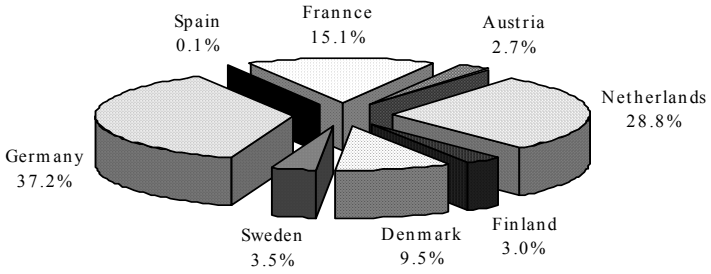


Fig 2.1 Distribution of EU starch quotas upon member states (2001)

The potato starch process is very simple and yields 160-180 kg of starch per ton of potatoes (Swinkels, 1990). The process is started by washing the potatoes, followed by peeling and grinding. During the process sodium disulfite is added to prevent a formation of brown polymers resulting from an oxidation of phenolic compounds. The grinded potatoes are pressed through rotating sieves. The fibers are retained and are discharged as potato pulp. The remaining starch slurry is separated by centrifuge or hydrocyclones and fine sieves. The purified starch slurry is used for the production of potato starch derivatives or is dewatering and dried. Therefore, the remaining by-product after starch processing are pulp and potato fruit juice (Knorr et al., 1977).

Since most European countries have an environmental regulation they require a purification of waste streams from potato factories regarding both the fruit water and the pulp. Several attempts have been made to de-water the by-products and to utilize them in the production of useful products. Normally, most of the raw pulp is immediately used for low value feed to avoid decomposition. Only small fractions of pulp are used for technical applications, which are summarized in Table 2.2. Proteolytic hydrolysis of potato pulp proteins is a method to obtain more valuable products as small peptides and amino acids. This is especially beneficial in starch production.

2.2 Enzymes

An enzyme is "*a protein with catalytic properties due to its power of specific activation*" (Dixon and Webb, 1979a). The activities of enzymes in the preparation and processing of food have been used for thousand years. The use of an enzyme catalyzed reaction may allow much shorter processing times under milder conditions. Therefore, they can replace a chemical reaction, which is harmful to human and the substrates, too. The primary source of industrial enzymes are microorganisms. Fifty percents of it originate from

fungi and yeast; 35 % from bacteria, while the remaining 15 % from either plant or animal origin (Rolle, 1998).

Table 2.2 Conventional applications of potato pulp (Mayer, 1998)

Treatment / Product	**Application**
Pulp supplement by potato proteins or other nitrogen-containing components (wet or partially dried and pelleted)	Cattle feed
Preparation of pectin or pectin–starch mixtures	Nutritional and other technical applications
Conversion into sugars and extraction of syrup	Treatment of potato chips and pommes frites (colour)
Hydrolysis for substrates used in fermentations	Alcohol production
Extraction of nitrogen-containing compounds from the liquid phase	Fertilizer
Dilution with water	Stabilizing factor in deep-drilling (lubicant)
Untreated substrate for growth of yeast	Vitamin B_{12} production
Untreated conponent of growth substrate	Biogas production

Enzymes have molecular weights ranging from about 12,000 to over one million daltons (Da) and demand physical space for movement and to be able to act on the much smaller functional groups in substrates. The basic properties of enzymes are normally determined by their protein nature. The various amino acid side chains are important factors, which influence the differences of the physical, chemical, and biological properties of proteins. Two of the most important chemical properties are charge and polarity. Some side chains have a carboxyl group at the free end, thus, are acidic amino acids, where as some have a nitrogen atom that can take up a proton, therefore, are basic amino acids. In addition, some side chains are aliphatic or aromatic hydrocarbon, and therefore poorly soluble in water but readily soluble in organic solvents (hydrophobicity of peptides and proteins). The physico-chemical properties of the natural amino acids are summarized in Table 2.3.

2.2.1 Enzyme Nomenclature

More than two thousand different enzymes are known, and it is likely that many more are awaiting discovery. Traditionally enzymes were simply assigned names by their investigator. In general, common names of enzymes are formed by taking the root of the word describing a substrate or the catalysis reaction of enzyme and adding a suffix (ending) "*–ase*".

For example, protease (or proteinase) refers to the enzymes which hydrolyze peptide bonds in protein molecules. As knowledge expanded, systems of enzyme classification became more comprehensive and complex. Thus, the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) formed the Enzyme Commission (EC) to develop a systematic numerical nomenclature of enzymes. The EC classification divided an enzyme into six different groups depending on the catalytic reaction type. Within each of these broad categories, the enzymes are further differentiated by a second number that more specifically defines the substrates on which they act, as summarized in Table 2.4. Individual enzymes in each subclass are further defined by third and fourth number. For example:

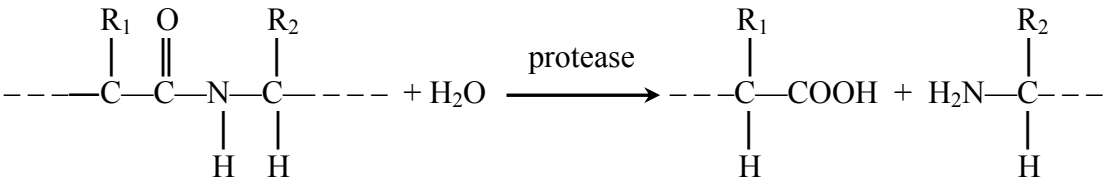
Name of enzyme (EC.1.2.3.4.)

EC – Enzyme Commission number system
1 – indicates the reaction catalyzed (1 – 6)
2 – indicates the general substrate or group involved
3 – indicates the specific substrate or coenzyme
4 – the serial number of the enzyme

2.2.2 Proteases

2.2.2.1 Introduction

Proteolytic enzymes or protease are globular and water soluble proteins. They catalyze the hydrolysis of peptide bonds in protein molecules. The general reaction catalyzed by protease can be illustrated as:



Proteases are the single class of hydrolytic enzymes, which are employed in industrial and medicinal applications, and basic research. The estimated value of the worldwide sales of industrial enzymes is $ 1 billion (Godfrey et al., 1996). Approximately 75 % of the industrial enzymes are hydrolytic enzymes. Protease represents one of the three largest groups of industrial enzymes and account for about 60 % of the enzymes sold worldwide (Rao et al., 1998).

Table 2.3 Physicochemical properties of the natural amino acids (Copeland, 2000)

Type of side chain	Amino Acid	Three-Letter Code	One-Letter Code	Mass of Residue in Proteins [a]	Accessible Surface Area ($A^{\circ 2}$) [b]	Hydro-phobicity [c]	pK_a of Ionizable Side Chain	pI at 25°C [d] $\frac{pK_1 + pK_2}{2}$	Occurrence in Protein(%) [e]	Relative Mutability [f]	Van der Waals Volume ($A^{\circ 3}$)
Carboxylic acid	Aspartate	Asp	D	115.09	150	-3.5	3.9	5.70	5.5	106	91
	Glutamate	Glu	E	128.14	180	-3.5	4.1	6.04	3.9	102	109
Basic side chain	Lysine	Lys	K	128.18	200	-3.9	10.8	5.02	7.0	56	135
	Arginine	Arg	R	156.20	225	-4.5	12,5	3.08	4.7	65	148
	Histidine	His	H	137.15	195	-3.2	6.0	5.63	2.1	66	118
Amide	Asparagine	Asn	N	114.11	160	-3.5		5.40	4.4	134	96
	Glutamine	Gln	Q	129.12	190	-3.5		6.04	6.2	93	114
S-Containing side chain	Cysteine	Cys	C	103.14	135	+2.5	8.4	6.00	2.8	20	86
	Methionine	Met	M	131.21	185	+1.9		5.74	1.7	94	124
Small neutral Side chain	Glycine	Gly	G	57.06	75	-0.4		5.91	7.5	49	48
	Alanine	Ala	A	71.08	115	+1.8		2.98	9.0	100	67
	Serine	Ser	S	87.08	115	-0.8		7.64	7.1	120	73
	Threonine	Thr	T	101.11	140	-0.7		6.06	6.0	97	93
Large hydrophobic Side chain	Valine	Val	V	99.14	155	+4.2		6.5	6.9	74	105
	Leucine	Leu	L	113.17	170	+3.8		6.30	7.5	40	124
	Isoleucine	Ile	I	113.17	175	+4.5		5.88	4.6	96	124
	Phenylalanine	Phe	F	147.18	210	+2.8		9.47	3.5	41	135
	Tyrosine	Tyr	Y	163.18	230	-1.3	10.1	5.68	3.5	41	141
	Tryptophan	Trp	W	186.21	255	-0.9		6.11	1.1	18	163
	Proline	Pro	P	97.12	145	-1.6		10.76	4.6	56	90

[a] Values reflect the molecular weights of the amino acids minus that of water.
[b] Accessible surfaces are for residues as part of a polypeptide chain. Data from Chothia C, *J. Mol. Biol.* 1975, **105**, 1.
[c] Hydrophobicity indices from Kyte J and Doolittle RF, *J. Mol. Biol.* 1982, **157**, 105.
[d] Merck & Co. (ed), *The Merck Index*, Nahway 1989, 11; *CRC Handbook of Chem. & Phys.*, Cleveland 1977, 58.
[e] Based on the frequency of occurrence for each residue in the sequence of 207 unrelated proteins. Data from Klapper MH, *Biochem. Biophys. Res. Commun.* 1977, **78**, 1018.
[f] Likelihood that a residue will mutate within a specified time period during evolution. Data from Dayoff et al., *Atlas of Protein Sequence and Structure*, Vol. 5, Suppl. 3, National Biomedical Res. Foundation: Washington 1978.

Table 2.4 Systematic basis of enzyme nomenclature[a]

First number (class)	Nature of reaction	Second number (first subclass)	Third number (second subclass)
1. Oxidoreductases	Electron transfer (oxidation-reduction)	Group in donor (D) oxidized	Acceptor (A) reduced
2. Transferases (synthases)	Chemical group transfer	Group transferred from D to A	Group transferred (further delineated)
3. Hydrolyase	Hydrolysis	Hydrolytic bond cleavages : ester, peptide, etc.	Substrate class : glycoside, peptide, etc.
4. Lyases	Bond-splitting	Nonhydrolytic bond cleavages : C – S, C – N, etc.	Group eliminated
5. Isomerases	Isomerization (changes in arrangements of atoms in molecules)	Type of reaction	Mix of S[b], reaction type chiral position involved in isomerization[c]
6. Ligases (synthetases)	Bond formation (joining together of two or more molecules	Bond synthesized : C – C, C – O, C – N, etc.	Substrate S_1, cosubstrate S_2, third cosubstrate is almost always nucleoside triphosphate

[a] Adapted from Parkin, 1993 and Copeland, 2000.
[b] S refers to substrate.
[c] Isomerases, racemases, epimerases, mutases, ligases (decyclizing, isomerizing).

2.2.2.2 Classification of proteases

Proteases may be classified using various criteria. They are usually classified according to three major criteria. The criteria currently used for the classification are: (i) their origin, i.e. animal, plant or microbiological, (ii) their mode of catalytic action, i.e. endo- or exo-activity, and (iii) on basis of their catalytic site. According to the NC-IUBMB, all proteases (peptide hydrolyases) are in subclass 3.4, which is further divided into 3.4.11 - 3.4.19, the exopeptidases, and 3.4.21 - 3.4.24, the endoprotease or proteinases. Endo-proteases are the most commonly used in food processing, but in some cases, their action is supplemented with exopeptidases.

2.2.2.2.1 Exopeptidases

The exopeptidases only act close to the ends of polypeptide chains. They are classified as amino- and carboxypeptidases based on their site of action by the N-or C-terminus, respectively.

- *Aminopeptidases* (EC 3.4.11 and EC 3.4.14). They act at a free N-terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide (Table 2.5). They are ubiquitous, but less readily available as commercial products, since many of

them are intracellular or membrane bound. Flavourzyme is commercially produced by fermentation of a selected strain of *Aspergillus oryzae* and contains both exopeptidase and endoprotease activities (Novo Nordisk). It can hydrolyze proteins, resulting in a taste improvement.

- Carboxypeptidases (EC 3.4.16 – EC 3.4.18). These enzyme act at C-termini of polypeptide chain and liberate a single amino acid or a dipeptide. They can be divided into three major groups, serine carboxypeptidases (EC 3.4.16), metallocarboxypeptidases (EC 3.4.17), and cysteine carboxypeptidases (EC 3.4.18), according to the nature of the amino acid residues at the active site of the enzymes. Many commercial proteases, in particular from fungi, contain significant amounts of carboxypeptidase activity.

Table 2.5 Classification of protease (Rao, 1998)

Protease	**Mode of action**[a]	**EC no.**
Exopeptidases		
Aminopeptidases	●-/-○-○-○-○—	3.4.11
Dipeptidyl peptidase	●-●-/-○-○-○—	3.4.14
Tripeptidyl peptidase	●-●-●-/-○-○—	3.4.14
Carboxypeptidase	——○-○-○-○-○-/-●	3.4.16 - 3.4.18
Serine type protease		3.4.16
Metalloprotease		3.4.17
Cysteine type protease		3.4.18
Peptidyl dipeptidase	——○-○-○-○-/-●-●	3.4.15
Dipeptidase	●-/-●	3.4.13
Omega peptidases	∗-●-/-○-○——	3.4.19
	——○-○-○-/-●-∗	3.4.19
Endoprotease	—○-○-○-/-○-○-○——	3.4.21 - 3.4.34
Serine protease		3.4.21
Cysteine protease		3.4.22
Aspartic protease		3.4.23
Metalloprotease		3.4.24
Endopeptidases of unknown		3.4.99
Catalytic mechanism		

[a] Open circles (○) represent the amino acid residues in the peptide chain.
Solid circles (●) indicate the terminal amino acids.
Stars (∗) signify the blocked termini.
Arrows (/) show the sites of action of the enzyme.

2.2.2.2.2 Endoproteases

They are characterized by their preferential action at the peptide bonds in the inner part of the polypeptide chain apart from the N- and C-terminus. The presence of the free amino or carboxyl group has a negative influence on these enzyme activity. Endoproteases are divided into four major subgroups based on their catalytic mechanism (Table 2.6).

- *Serine proteases*. They are characterized by the presence of serine residue at their active site. They are the most common class of protease, being produced by bacteria, eukaryotes, and viruses and are found in the exopeptidase, endoprotease, oligopeptidase, and omega peptidase groups. These enzymes are generally active at neutral and alkaline pH, with optimum pH between 7-11. They have broad substrate specificities. Their molecular masses range between 18-35 kDa, except those from *Blakeslea tripora*, which has a molecular mass of 126 kDa (Govind et al., 1981). The isoelectric points of serine proteases are between pH 4-6. They can be divided into a number of subfamilies based on structural similarities. Serine alkaline proteases are produced by several bacteria, molds, yeasts, and fungi. They hydrolyze a peptide bond which has tyrosine, phenylalanine, or leucine at the carboxyl side of the splitting bond. The optimum pH of this group is around pH 10 and the isoelectric point is around pH 9. Their molecular masses range between 15-30 kDa. They are the largest subgroup of serine proteases. The second largest are bacterial subtilisins, which enjoy the greatest industrial significance. Subtilisins which are produced by selected *Bacillus spp*. have found widespread application as detergent additives.

- *Aspartic proteases*. They are commonly known as acidic proteases that contain an aspartic acid residue at the catalytic site. Most of them show maximal activity at low pH (pH 3- 4) and have isoelectric points in the range of pH 3-4.5. Their molecular masses are in the range of 30-45 kDa. They can be broadly divided into two groups, (i) pepsin-like enzymes produced by *Aspergillus, Penicillium, Rhizopus*, and *Neurospora* and (ii) rennin-like enzymes produced by *Endothia* and *Mucor spp*.

- *Cysteine* protease. They are widely distributed in nature and are characterized by the presence of cysteine and histidine residue at the active site, which forms a catalytic dyad. The best known enzyme of this group is papain. These proteases are generally active under reducing conditions and tend to show optimum activity at neutral pH values.

- *Metalloproteases*. They are the most diverse of the catalytic types of protease (Barett, 1995). They contain an essential divalent metal ion, usually zinc (Zn) for their activity, and have optimum activity around neutral to alkaline pH. Their stability is increased in the presence of Ca^{2+} and inhibited by strong chelating agents, such as EDTA, by removing the zinc atom. The best known enzyme of this group is microbial thermolysin, a very heat-stable neutral protease.

Table 2.6 List of proteases for food proteins hydrolysis[a]

Type of proteases	Common names	Source	pH-range	Preferential specificity[b]
Serine protease				
Animal	Trypsin	Ox, Pig	7 – 9	Lys-, Arg-COOH
	Chymotrypsin		8 – 9	Phe-, Tyr-, Trp-COOH
	Elastase		6 – 8	Ala-COOH
Bacteria	Substilisin Carberg, Alcalase[c]	*Bacillus licheniformis*	6 – 10	Broad specificity, mainly hydrophobic -COOH
	Substilisin BPN, Substilisin NOVO	*Bacillus amyloliquefaciens*	6 – 10	Broad specificity, mainly hydrophobic -COOH
Aspartic protease				
Animal	Pepsin, Pepsin A	Ox, Pig	1 – 4	Aromatic-COOH and $-NH_2$, Leu-, Asp-, Glu-COOH
	Chymosin, rennin	Calf	3 – 6	Rennet specificity
Fungal	Chymosin-like	*Mucor pusillus* *Mucor miehei* *Endothia parasitica*	3 – 6	Rennet specificity
	Aspergillo peptidase A (pure aspartic protease)	*Aspergillus saitoi*	2.5 – 5	Pure aspartic protease: aspepsin Mixed preparations: broad specificity
	Newlase, SumyzymeRP	*Rhizopus sp.*	3 – 5	As pepsin
Cysteine protease				
Plant	Papain, pure	Papaya latex	5 – 7	Lys-, Arg-, Phe-X-COOH
	Papain, crude		5 – 9	Broad specificity
	Bromelain	Pineapple stem	5 – 8	Lys-, Arg-, phe-, Tyr-COOH
	Ficin	Ficus latex	5 – 8	Phe-, Tyr-COOH
Metalloprotease				
Animal	Carboxy peptidase A	Pancreas	7 – 8	Terminal a.a. at C-terminus of peptide, except Pro, Arg, Lys
Bacteria	Neutral protease Neutrase	*Bacillus amyloliquefaciens*	6 – 8	Leu-, Phe-NH_2 and other
	Neutral protease Thermolysin	*Bacillus thermoproteolyticus*	7 – 9	IIe-, Leu-, Val-, Phe- NH_2
Technical preparations				
Mixture of papain, chymopapain and lysozyme	Crude papain	Papaya fruit	5 – 9	Broad specificity
Mixture of trypsin, chymotrypsin, elastase and carboxypeptidase A or B	Pancreatin	Pancrease (ox, pig)	7 – 9	Very broad specificity
Mixture of serine-, aspartic-, metallo-protease	Veron P, Sumyzyme LP Biozyme A	*Aspergillus oryzae*	4 – 8	Very broad specificity
Mixture of endo- and exo-protease, active at alkaline and neutral pH	Pronase	*Streptomyces griseus*	7 – 9	Very broad specificity

[a] Data from (Adler-Nissen, 1993; Whitaker, 1994; Uhlig, 1998).
[b] Terminal amino acid after cleavage.
[c] Commercial preparations.

2.3 Enzymatic protein hydrolysis

Enzymatic protein hydrolysis is the degradation of proteins into peptides and/or amino acids by proteolytic enzymes. The proteins hydrolyze in three subsegment phases: (i) the formation of Michaelis complex of substrate (the original peptide chains) and enzyme, (ii) the peptide bond is cleaved resulting in the liberation of one peptide, and (iii) the remaining complex is split off the other peptide after nucleophilic attack by a water molecule. Thus, their steps can be simplified as follows:

$$E + S \underset{K_{-1}}{\overset{K_1}{\rightleftharpoons}} ES \xrightarrow{K_2} EP + H\text{-}P' \xrightarrow[H_2O]{K_3} E + P\text{-}OH + H\text{-}P'$$

where: E – Enzyme

S – Substrate

P and P′ – resulting peptides

K – reaction velocity constants

Due to the protein hydrolysis the molecular properties of proteins are changed, namely decrease of molecular weight, increase of charge, exposure of hydrophobic groups and exposure of reactive amino acid side-chains (Nielsen, 1997). These molecular characterization can be detected with several analytical methods, such as size exclusion chromatography (SEC), reverse-phase chromatography (RPC), and degree of hydrolysis (DH) etc. which reflect one or several molecular properties.

The degree of hydrolysis is the most commonly used parameter describing the result of a hydrolyzation process, used as an indicator for the extent of hydrolysis. This parameter represents the proportion of peptide bonds hydrolyzed and is calculated according to this equation:

$$\%\ \mathrm{DH} = \frac{h}{h_{tot}} \times 100\ \%$$

where: h – the number of peptide bonds hydrolyzed.

h_{tot} – the total number of peptide bonds presented in the parental protein.

Most of h and h_{tot} are expressed in meq/g. The h_{tot} of the protein is calculated from the protein amino acid composition (Adler-Nissen, 1986). During the hydrolyzation process a new carboxyl and a new amino group are released for each cleaved amind bond. Therefore, the number of hydrolyzed peptide bonds can be deduced from the determination of the number of newly formed C- and/or N-terminus groups in the hydrolysates.

The amount of released α–amino groups can be detected by using specific reacting regents with amino groups, yielding derivatives that can be detected spectrophotometrically. The reagents generally used ninhydrin, σ-phthalaldehyde (OPA), and trinitrobenzene-sulphonic acid (TNBS). The DH results obtained from OPA and TNBS showed a high correlation, whereas a determination with ninhydrin reagent resulted in much lower DH values (Panasiuk et al., 1998). The OPA method is superior to the TNBS method since it was faster and more accurate (Mant, 1992). Thus, in this study the measurement of DH with the OPA method was used.

2.4 Chromatographic Methods

Chromatography is a term which encompasses a number of techniques used to separate components of a mixture into their individual units or compounds as well as to determine their percentage within the entire mixture. A variety of chromatographic methods and techniques exist, and the proper technique depends upon what is to be separated and the physical/chemical properties of each of the components involved. Normally, the separation bases on the difference in affinity of a sample component towards a mobile phase in comparison to a stationary phase. The mobile phase and stationary phase can both be liquid or gas. In liquid chromatography the compounds to be separated are dissolved in a solvent, which acts as mobile phase while the column (and/or the packing) acts as stationary phase. The quality of the stationary phases for the HPLC (High Performance Liquid Chromato-graphy) is determined by their physical and chemical properties. The physical properties, such as porosity, specific surface area, particle shape and size, and pore size, greatly determine the efficiency of a packing. The chemical properties, which are a result of substrate properties and the applied surface bonding chemistry, form the basis of retention and selectivity.

Several types of liquid chromatography techniques have been successfully used in research and industry (i.e. different types being used for different purposes). HPLC is one of the most widespread liquid chromatography techniques that uses high pressured liquid flowing through the column to achieve a high resolution of separation (Nice, 2000). Because of its dynamic nature, HPLC has found use for a wide range of applications in many industries.

2.4.1 Reversed Phase High Performance Liquid Chromatography (RP-HPLC)

Nowadays, RP-HPLC is the most popular mode of analytical liquid chromatography. It is used in analytical and preparative applications in the area of biochemical separation and purification. Approximately 90 % of all analytical separations of low molecular weight compounds are carried out by using a RP-HPLC. In the analysis of intermediate molecular weight compounds such as peptides, this technique still plays an important role because of two reasons: (i) The secrets of the separation principle are mostly revealed. And (ii) an acceptable separation result of a great variety of samples, containing non-polar, polar, and even ionic compounds, is possible without technical complications (Neue, 1997). The separation mechanism depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilized hydrophobic ligand, i.e. the stationary phase. Peptides and proteins are adsorbed onto the hydrophobic surface of the column, and remain there until the concentration of the organic modifier is high enough to elute the molecules from the hydrophobic surface. The elution order is related to the increasing hydrophobic nature of the solutes. The more soluble a solute is in water or the more hydrophilic the solute, the faster it will be eluted. Thus, molecules that have some degree of hydrophobicity, such as peptides and proteins, can be separated by this method with excellent resolution and recovery.

2.4.1.1 Stationary phases

The heart of liquid chromatograph is a column, where the compounds of the sample are separated. A suitable column should provide adequate separation efficiency and separation selectivity, stability, reproducibility, and should have a sufficient lifetime. Selecting a column for a particular application, means choosing between two basic alternatives: (i) the packing chemistry which is the most suitable for the separation, and (ii) the physical properties of the column, especially particle size and column dimensions (Neue, 1997). In Table 2.7 the physical properties of HPLC column and separation conditions are shown. The determination of a correct packing material is a primary task, since it is an interaction between sample constituents and packing material. However, the choice of column dimensions and particle size are also important, since it influences speed of the analysis, resolving power, column back pressure, detection ability, and solvent consumption per analysis. In general, the column plate number, the pressure drop across the column, and the separation time at a constant flow rate are directly proportional to the column length. Any amount of sample load can be separated without column overloading if column diameter is increased appropriately.

Table 2.7 Physical properties of HPLC column and separation condition (Jandera, 2000)

Column type	Length (cm)	Internal diameter (cm)	Particle size (μm)	Flow rate (ml / min)	Amount of sample per separation (g)
Conventional	6 - 25	0.3 - 0.46	3 - 10	1 - 3	10^{-10} - 10^{-4}
High speed	2 - 5	0.3 - 0.46	1.5 - 5	2 - 5	10^{-10} - 10^{-4}
Microbore	10 - 50	0.05 - 0.21	3 - 10	0.02 - 0.2	10^{-12} - 10^{-5}

Generally in RP-HPLC, the columns are composed of hydrocarbon alkane chains, which are covalently attached to silica particles. These chains range from C4 (n-butyl) to C18 (n-octadecyl) carbon atoms in length. These hydrophobic groups are responsible for the specific chromatographic character of the column. Since elution from the column is a function of the hydrophobicity, the longer hydrocarbon chain columns are better used for small, highly charged peptides, and, large hydrophobic peptides elute greater using short chain hydrocarbon supports (Neue, 1997).

2.4.1.2 Mobile phases

The second major component in HPLC is a mobile phase. This phase refers to the solvent being continuously applied to the column. It acts as a carrier for the sample solution. Then a sample solution is injected into an assay mobile phase through the injector port. With a sample solution flowing through a column with the mobile phase, the migration of the components in the solution depend on to the non-covalent interactions of the compounds with the column. The chemical interactions of the mobile phase and of the sample with the column determine the degree of migration and separation of the components contained in the sample. For example, the samples, which have stronger interactions with the mobile phase than with the stationary phase, will elute faster from the column, and have a shorter retention time. The changing of the selectivity properties of the phase system influences the separation most. This can be done by using another method (e.g. normal or reversed phase), or another stationary phase (e.g. octadecyl or phenyl silica), or another mobile phase. The mobile phase can be changed in order to manipulate the interactions of the sample and the stationary phase. There are several types of mobile phase (solvent) to choose from. There are three fundamental factors that have to be considered: (i) physical properties of the solvent, (ii) the chemical properties of the solvent (especially with respect to system compatibility and safety aspects), and (iii) the effects of these properties on the chromatographic process (e.g. system operation, chromatographic separation, detection limits, and analytical reproducibility) (Sadek, 2002). Generally, the mobile phase in RP-HPLC contains water and one or more water-soluble organic solvents. The most widely used organic solvents in RP-HPLC are summarized in

Table 2.8. Binary mobile phase is used more often than those containing more than one organic solvent in the water. The elution strength increases if the polarity of the organic solvent decreases or the concentration in the aqueous-organic mobile phase increases.

Table 2.8 Properties of some selected solvents for RP-HPLC[a]

Solvent	UV cut-off[b] (nm)	Boiling point (° C)	Viscosity (cP at 20 °C)	Polarity (p´)	Strength (ε)	Comments
Acetonitrile	190	82	0.36	5.8	0.65	More powerful denaturant than alcohols. Toxic.
Methanol	205	65	0.60	5.1	0.95	
Ethanol	210	78	1.20	4.3	0.85	
1-propanol (n-propanol)	210	98	2.26	4.0	0.82	Viscous
2-propanol (iso-propanol)	210	82	2.30	3.9	0.82	Viscous
Water	< 190	100	1.00	10.2		

[a] Data adapted from Snyder, 1979 and Amersham pharmacia biotech.
[b] Absorbance is approximately 1 for HPLC-grade solvent at this wavelength.

Normally, water, methanol, acetonitrile (ACN), and isopropanol (IPA) are often used as a mobile phase (Sadek, 2002). Water is used as a part of mobile phase in nearly all of reversed phase separations. Since it is ubiquitous and very popular solvent used by almost every chromatographer. The hydrophobic strength of these organic solvents decreases in the order of IPA > ACN >> methanol (Hartman et al., 1986). Therefore, in 90-95 % of all published essays of peptides separation, ACN was used as a mobile phase due to several advantageous characteristics of this solvent, including low viscosity, high volatility, low UV transparency, and high degree of selectivity (Wilson et al., 1981). Although ACN is commonly used as mobile phase, alcohols, particularly IPA, are occasionally used to improve the solubility of hydrophobic peptides. It is known as a "universal solvent" because of it is miscible with water as well as with a wide selection of polar and nonpolar water-immiscible solvents (e.g. hexane or dichloromethane). Besides that, IPA can be effectively used to clean up and regenerate RP-columns, since this solvent can solubilize a wide range of solutes (Sadek, 2002).

2.4.1.3 Method development and optimization of conditions in RP-HPLC

The method development requires a clear goal of the separation. The primary objectives might be: (i) resolution, detection, characterization, or quantification of one or

more substances in a sample, so that the complete separation is not necessary, (ii) complete resolution, characterization and quantification of all sample components, (iii) isolation of purified sample components for spectral identification or for other assays. Other points that have to be considered include the required sensitivity, accuracy, precision, character of sample matrices, expected frequency of analysis, and the available HPLC equipment. There are many approaches of method optimization. The aim of method development and optimization is to find the best separation conditions for the reliable measurement of the amounts of individual components in the mixture. The most common strategy for developing methods in practice is still trial and error combined with empirical knowledge and pragmatic rules. It depends on a large number of factors, including physical and chemical properties of the sample mixture and of the mobile and stationary phases (Grize et al., 1994).

The first step in method development is selecting an adequate column. Two important facts should be kept in mind for a survey of the properties of stationary phase and their applications in peptide analysis: (i) The selection of the stationary phase is a part of the approach to the whole analytical problem and can not be considered separately from the choice and optimization of the mobile phase and the experimental conditions. (ii) The physico-chemical character and properties of stationary phase information; these detail are often difficult or impossible to obtain, since they tend to be a trade secret (Smith et al., 1987). Because of the complexity of the mixtures to be separated, a very high resolution is required. The slowness of mass transport and sorption/desorption processes is the most important reason for poor resolution. This can be improved by working at elevated temperatures and by choosing stationary phase types that permit rapid solute transport. In addition, the large peptide molecules with many active sites behave differently from small peptide molecules. The capacity factors change much faster when the content of the organic modifier in mobile phase change and the steepness of this dependence increases with the increase of the molecular size of the solute. Thus, gradient elution have to be employed and the conditions carefully optimized. Then the resolution does not greatly depend on the column length and the flow rate of mobile phase (Kato et al., 1990).

After choosing an appropriate column the next step of method development and optimization involve mobile phase composition and the choice between isocratic or gradient elution. Many complex samples contain compounds which differ widely in retention factor (k), so they cannot be separated with isocratic elution. This problem can be solved by gradient elution. Gradient elution is the most widely used technique in HPLC since the composition of the mobile phase is changed during the chromatographic run. Slow increase of

the mobile phase elution strength in gradient elution allows a decrease of the retention factor. In RP-HPLC the elution strength increases if the polarity of the organic solvent decreases or its concentration in the aqueous-organic mobile phase increases. Hence, the concentration of organic solvent is lower in the initial mobile phase (mobile phase A) than it is in the final mobile phase (mobile phase B). Generally, it takes a longer running time than isocratic elution since the column should be re-equilibrated to the initial gradient conditions after each run. However, because of the separation power of gradient elution, it is required or preferred for the separation of many samples, not only these with a wide *k* range but also for samples composed of large molecules, like biopolymers or samples containing late-eluting interference that would overlap peaks in following chromatograms.

2.4.1.4 Effect of temperature on the separation

In liquid chromatography the heat transfer of solute between mobile and stationary phase is much smaller than in gas chromatography. Thus, change in temperature has much less effect on the degree of retention and resolution. Nevertheless, an increase in temperature would reduce the mobile phase viscosity. Since mass transport of solute between the mobile phase and the stationary phase is a diffusion-controlled process, decreasing the mobile phase viscosity generally produces an increased rate of mass transfer and increased solute solubility. Therefore, decreasing the capacity factor, results in sharper peaks, giving better resolution and shorter analyzing time (Braithwaite and Smith, 1996). Accordingly, temperature regulation can be used for optimizing the resolution. This is very simple and convenient, since it only requires a column thermostat which can be connected to the HPLC system to control temperature automatically. Temperature optimization is usually less effective in improving the quality of HPLC separation than varying the composition of mobile phase, but might be very useful if used in combination with the control of the mobile phase composition or the gradient elution (Zhu et al., 1996). Temperature can have an effect on RP chromatography, especially for low molecular weight solutes like short peptides and oligonucleotides. They are sufficiently stable at the elevated temperature. The disadvantages of temperature increase in solvent and sample components are: (i) They are more likely to decompose. (ii) The vapour pressure of the solvent rises. Then increasing the risk of bubbles in the detector, which in turn produce an uneven baseline, ghost peaks or even complete light absorption. (iii) All ion equilibria in the mobile phases are temperature dependent. It is more difficult to control at elevated temperature. Therefore, the reproducibility may be poor if thermostating is not adequate. And (iv) the solubility of silica greatly increases in all mobile phases when the temperature rises, too (Meyer, 1999).

2.4.2 Size Exclusion Chromatography (SEC)

SEC or gel filtration chromatography (GFC) is a widely used and well documented method to analyze the molecular weight of peptides from protein hydrolysates. It is a non-interactive technique that separates solutes by their differences in shape and molecular size, and it is often used as a first step in the isolation of peptides and proteins from the crude sample. The main applications of this technique in relation to the peptides study are: (i) purification and analysis of purity, (ii) estimation of molecular weight, and (iii) study of interactions, either self-association or with other molecules (Irvine, 1997). It continues to be an efficient separation methods in peptide purification.

Results from SEC are usually expressed as elution profiles or chromatogram charts show the variation of concentration (typically in the terms of UV absorbance) of the sample components as they elute from the column in the order of their molecular size. These molecules larger than pores of the packing elute first with the solvent front and are completely excluded. Molecules of intermediate sizes between the completely excluded and the retained pass through the pores of the matrix according to their sizes. Small molecules, which freely pass in and out of the pores, are retained. Therefore, different sizes of peptides and proteins have different elution volumes and retention times. For similar structural molecules, the larger the molecular weights or sizes are, the earlier they will be eluted out. Hence, larger molecules will elute earlier and the smaller ones later.

The SEC method development requires of selecting a mobile phase compatible with the sample type, i.e. the columns must have sufficient pore volume and an adequate range of pore sizes to resolve the sample's molecular weight distribution. Thus, the selection of the packing matrix in SEC is selected from the relative size of peptides to be separated (Malawer, 1995). The used matrixes have a closely controlled pore size, with a high chemical and physical stability. They are hydrophilic and inert to minimize chemical interactions between the solutes (peptides) and the matrix itself. Many matrixes retain a residual charge owing to, for example, sulfate groups in agarose or carboxyl residues in dextran. The most widely used packing materials for polypeptide separation have been of two main types (Gooding and Regnier, 1990): (i) Inorganic, based on silica particles coated with a hydrophilic outer layer to prevent protein adsorption. They are limited to the pH range 2-8, e.g. TSK SW and Synchropak GPC. And (ii) organic polymeric particles cross-linked to introduce rigidity. However, cross-linking also decreases pore volume and introduces a hydrophobic character, e.g. cross-linked dextan or agarose (Sephadex or Sepharose), polyacrylamide beds (Bio-Gel), and dextran derivatives (Sephacryl) (Hostettmann et al., 1997). These tend to be stable over a wider pH range than the silica-based support.

Because some of the biomolecules have a strong affinity to the surface of stationary phase, the mobile phase should be chosen carefully to fit certain criteria: (i) It must completely dissolve the sample in a continuous solution phase. (ii) Its viscosity has to be low enough for the operation SEC system at the normal pressure range. (iii) And it has to effectively prevent the solute molecules in the sample from interacting energetically with the stationary phase (e.g. adsorption). The failure even one of these criteria results in the system's inability to characterize the sample properly (Malawer, 1995). SEC operates by isocratic elusion, i.e. with a fix composition of the mobile phase. It is a great advantage that there is no need to re-equilibrate the column between the runs compared with other chromatographic methods such as ion exchange or reversed phase, which require a change in the mobile phase composition during the run (gradient elusion).

2.5 Peptide Characterization

Peptides mixtures arising from the enzymatic hydrolysis of proteins are usually fractionated and characterized by a combination of the chromatographic methods such as ion-exchange chromatography, SEC, and RP-HPLC. Appropriate combinations of these methods will normally yield a homogeneous product, but their costs, detection limits, analyses times, and yields vary (Hughes et al., 1979). The combination of liquid chromatography and mass spectrometry (LC/MS) offers one of the most powerful techniques in modern times for the characterization of peptide and protein molecules through the determination of molecular weight and structure. Mass spectrometry (MS) is an instrument that can ionize a sample and measure the mass-to-charge ratio (*m/z*) of the resulting ions. MS can not only give qualitative and quantitative information on the elemental, isotopic, and molecular composition of organic and inorganic sample but it also provides an excellent selectivity by detecting the signals related to the molecular weight of the analyte and reduces the analytical cycle time (Deters et al., 2002). It has three basic functions: (i) the generation of ions in the gas phase, (ii) the separation of the gas phase ions according to their *m/z*, and (iii) the detection of the separation ions. The MS instrument must be connected to a computer system to process and record the data and a vacuum pump to control the pressure within the mass spectrometer. The choice of ion source generation depends on the sample properties and the degree of ionization and fragmentation desired (Finehout and Lee, 2004). In general, the two most commonly used methods are electrospray ionization mass spectrometry (ESI-MS) and matrix-assisted laser desorption ionization (MALDI) coupled to time-of-flight analyzer (TOF). These techniques are fast, simple, highly accurate, and subpicomole sensitive (Cotter, 1992; Chait and Kent, 1992).

2.5.1 Electrospray Ionization mass Spectrometry (ESI-MS)

ESI-MS is a gentle technique for charging a substance, and this process predominantly occurs by transferring one or more protons onto the analyte from proton-bound (protonated) reagent/solvent molecules in the liquid phase. This technique has performed most efficiently for the separation and characterization of enzymatic fragment mixture of proteins (Banks, 1996; Chang et al., 1994; Mock et al., 1993). There are two major steps in the process of ESI-MS: (i) the formation of gas phase ions from ions in the solution, and (ii) the analysis of the produced gas phase ions in the mass analyzer. The peptide or protein solution is injected into the capillary of the mass spectrometer at high voltage (2-8 kV). A fine spray of charged droplets is produced. When a positive potential is applied to the tip of the spraying needle (the emitter), positively charged molecules will accumulate at the tip of the capillary, and negative charged molecules will oxidize (Fig. 2.2). The solution being sprayed emits droplets containing excess charge and forming a conical distribution (Taylor cone). A heated drying gas, e.g. nitrogen, or heated capillary can be used to assist evaporation of the solvent sheath from the ion. Finally, the desolvated, multiply charged ion is introduced into the high vacuum analyzer of the mass spectrometer for analysis (Kebarle, 2000).

The multiplicity of organic functional groups in a biological molecule enables for the presence of multiple charges, because these functional groups can attract protons. This characteristic allows ESI-MS to contain more than one mass spectral peak for a biological analyte. In most cases, multiple peaks will differ only by the number of protons attached to the biological species. For peptides and proteins in the positive-ion mode, (i) the terminal amine (NH_2), (ii) the amine functional group in the side chains of lysine and arginine, and (iii) the nitrogen atoms in the histidine residue are the preferred sites of protonation because of their relatively high proton affinities (Stryer, 1994). Therefore, this technique continously produces multiply charged ions of the type $[M + nH]^{n+}$, with M being the molecular mass of the molecule and n being the number of charges. The series of $[M + nH]^{n+}$ ions allows a very precise determination ($\leq 0.01\%$) of M via a simple algorithm.

2.5.2 Matrix-Assisted Laser Desorption Ionization (MALDI)

MALDI is first introduced in 1988 by Karas and Hillenkamp (Karas and Hillenkamp, 1988). It has become a widespread analytical tool for peptides, proteins, and most other biomolecules (oligonucleotides, carbohydrates, natural products, and lipids). The comparison of the principle characteristics of ESI and MALDI methods is shown in Table 2.9. In MALDI analysis, peptide or protein is first co-crystallized with a UV-absorbing weak organic acid matrix on the metal plate. The three most common matrixes are: (i) α–cyano-4-hydroxy-

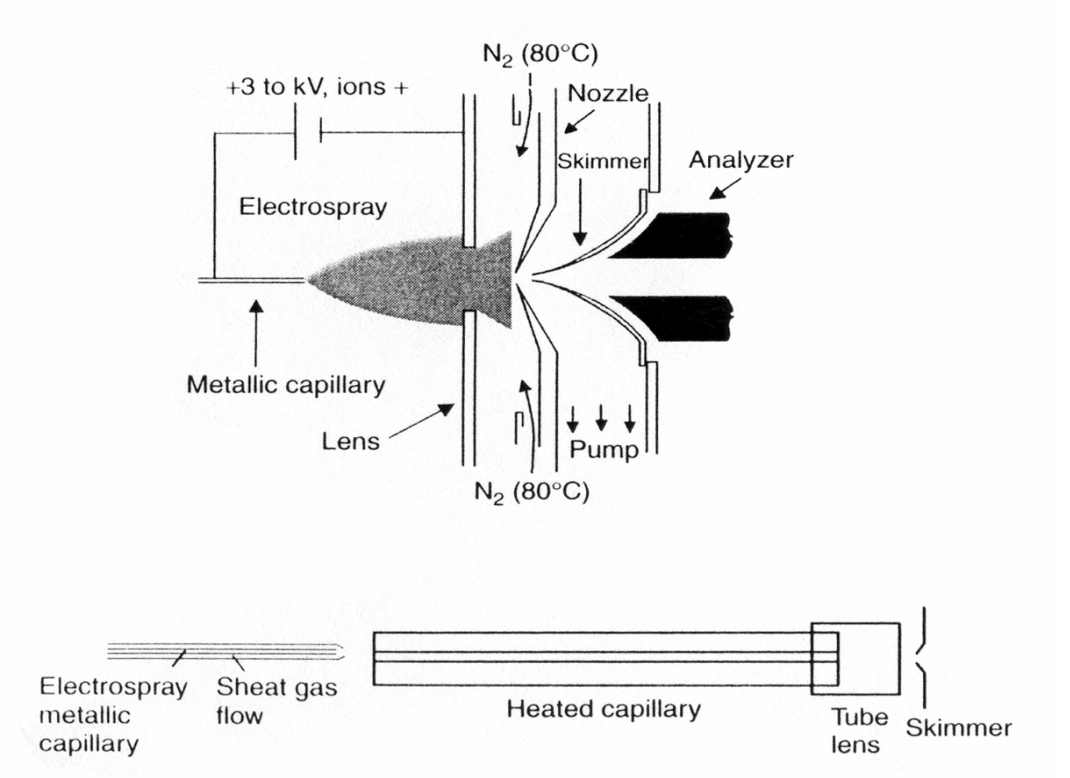


Fig. 2.2 Conceptual scheme of electrospray ionization (ESI) process in positive ion mode (Hoffmann and Stroobant, 2002)

cinnamic acid (α–cyano is mostly used for the analysis of peptides and small proteins, peptide mapping, and post source decay (PSD) analysis), (ii) 3,5-dimethoxy-4-hydroxy-cinnamic acid (sinapinic acid (SA) is mostly used for large proteins), and (iii) 2,5-dihydroxy-benzoic acid (DHB is a more generally applicable matrix for both peptides and proteins, but the crystal are not as homogenous as with α–cyano and SA) (Hillenkamp et al., 1991).

Upon irradiation with laser light of appropriate wavelength and under high voltage (~ 20 kV), the singly charged ions are produced (Karas et al., 2000). The laser is tuned to the absorption maximum of the matrix. The sample ions are preformed in the condensed phase in sufficient quantities. The matrix absorbs some of the laser pulse energy, thus minimizing sample damage. The sample ions and matrix molecules gain enough kinetic energy and are ejected into a gas phase (Fig. 2.3). Therefore, the matrix plays a key role in strongly absorbing the laser light energy and indirectly causing the sample to vaporize. It also serves as a proton donor and receptor, acting to ionize the sample in both positive and negative ionization modes, respectively (Hillenkamp et al., 1986).

MALDI has been coupled to many different mass analyzers, e.g. a linear time-of-flight (TOF), a TOF reflection, and a Fourier transform mass analyzer. The TOF analyzer seems the best suited for the pulsed ion production. It can in principle handle an unlimited mass range,

Table 2.9 Comparison of ESI and MALDI methods (Zybailov and Washburn, 2005)

	ESI	**MALDI**
Principle of action	Uses electric field to produce sprays of fine droplets, as the droplets evaporate, ions are formed	Uses laser pulses to desorb and ionize analyte molecules, co-crystallized with a matrix on a metal surface
Ions formed	Multiple charged (The larger the analyte molecule, the more likely it acquires multiple charges)	Singly charged
Mass range	> 100 kDa	> 100 kDa
Resolution	~2500 (with ion trap/quadrupole mass analyzers)	~10000 (with TOF mass analyzers and ion reflectors)
Typical application	10^{-15} mole LC/MS of peptide mixtures, tandem MS, protein identification by comparing experimental MS/MS spectra produced from protein databases	10^{-15} mole Analysis of spots on 2D-PAGE, determination of molecular weight, protein identification by peptide mass fingerprinting

and can be built with relatively low costs. TOF analysis is based on accelerating a set of ions to a detector where to all of the ions is given the same amount of energy. Because the ions have the same energy, yet a different mass, the ions reach the detector at different times. The smaller ions reach the detector first because of their greater velocity, while the larger ions take longer due to their larger mass.

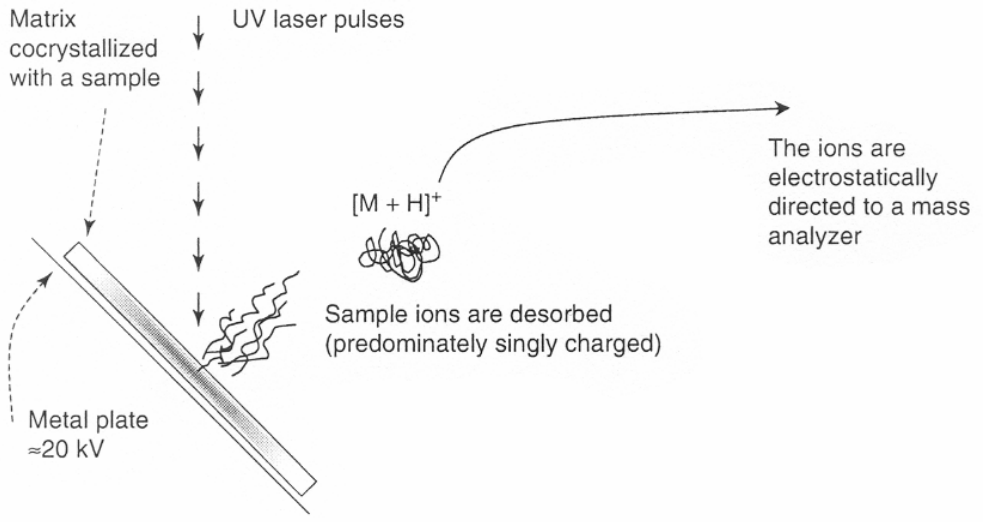


Fig. 2.3 Conceptual scheme of matrix-assisted laser desoption ionization (MALDI) (Zybailo and Washburn, 2005)

While the MALDI mass spectrometer is a powerful tool for the accurate mass determination of peptide mixtures, obtaining the precise mass measurement is highly dependent upon the sample and the sample preparation. In general, ionization occurs through proton transfer or cationization. The ionization depends critically on the matrix-analyte combination, but is not critically dependent on the number of acidic or basic groups of the analyte (Beavis et al., 1990). Acquiring optimum MALDI data depends on the choice of suitable matrixes and solvents, the functional and structural properties of the analyte, sample purity, and on how the sample is prepared on the MALDI sample plate. Because the solvent or matrix is the medium by the analyte will be transported to the gas phase and provides the conditions that make ionization possible. The excessive salt (> 10 mM) contamination in the sample will lead to reduced sensitivity. Dialysis, and RP-HPLC or exchange chromatography are useful methods for purifying sample contaminants prior to mass spectral analysis.

CHAPTER 3 AN IMPROVEMENT OF POTATO PULP PROTEIN HYDROLYZATION PROCESS BY THE COMBINATION OF PROTEASE ENZYME SYSTEMS

3.1 Introduction

The increasing human population over the last decades has greatly influenced the demand for food products. Potato (*Solanum tuberosum*) is a major world crop of which more than 300 million tons are produced worldwide annually. It is the most important vegetable in European countries today. Potatoes are used for several purposes, including human consumption, industrial processing (potato starch, alcohol etc.), and recultivation (Feustel, 1987; Tallburt, 1987). Fresh potato contains about 17-21 % starch, 0.5-1.2 % proteins, mineral compounds, organic acids, and ash (Liu, 2002; Smith, 1977). Therefore, it can be used as a raw material for starch production. Furthermore, the tuber contains a higher proportion of the essential amino acid lysine (Lys) than most cereal protein, but is deficient in the sulphur-containing amino acids, methionine (Met) and cysteine (Cys) (Ralet and Gueguen, 1999; Knorr, 1978; Kapoor et al., 1975).

Potato starch was first produced in Germany since the end of seventeenth century. During the processing low-cost by-products with regard to both potato fruit water and potato pulp were produced. After grinding the potato and washing out the starch potato pulp remains. On a dry matter basis the pulp contains 74 % of protein in comparison to the content in potato tuber. Since most European countries have strong environmental regulation for industrial waste water they require a purification of waste streams from potato factories regarding both the fruit water and the pulp. Several attempts have been made to dehydrate the by-products and to utilize them for differential purposes. Normally, most of the raw pulp is immediately used for low value feed to avoid decomposition (Lisinska and Leszczynski, 1989). Only small fractions of pulp are used for technical applications (Mayer, 1998). Thus, an increased utilization of potato proteins by-products would be economically interesting for potato starch factories (Van Koningsveld et al., 2001; Knorr, 1982; Knorr et al., 1977). Several attempts have been done to receive more valuable and useful products. Enzymatic hydrolysis is one of the main methods for altering food proteins (Hrčková et al., 2002; Franek et al., 2000). There are many commercially available enzymes that can be used. Proteases are commonly used to partially hydrolyze proteins under mild conditions with high and reproducible specificity. They can be classified based on their origin, i.e. animal, plant, or microbial origin, their mode of catalytic action, i.e. endo- or exo-activity, or on their catalytic site (Rao et al., 1998). Endo-

proteases cleave peptide bonds in the inner regions of the polypeptide chain, whereas exo-proteases act only close to the end terminus of polypeptide chains, either at the C-terminus (carboxypeptidases) or at the N-terminus (aminopeptidases) (Adler-Nissen, 1993).

During an enzymatic hydrolyzation process proteins are cleaved to smaller molecules, namely smaller peptides and free amino acids. Hence, the nutritional quality and safety of the products improves. New products could be generated and alternative applications for several agricultural products could be realized. The economical success of the new products is due to the fact that they meet certain technical quality requirement and can be produced and treated at competitive costs. Enzymatic hydrolysis offers an advantage over the chemical hydrolysis process of plant protein.

During the past decade utilization of plant protein from soybean, nut, wheat, and oil plants has increased tremendously for nutrition and economic reasons. They contain large amount of high quality protein and are being utilized as excellent source of plant based protein (Deparis et al., 2003; Korhonen and Pihlanto, 2003; Braudo et al., 2001; Adu-Amankwa et al., 1993). Because of a lack of knowledge about the effective process for modifying and the utilizing of potato pulp protein, thus the aim of this study was to find an appropriate enzyme combination for the production of PP protein hydrolysates with a high degree of hydrolysis and a quality improvement. PP was enzymatically hydrolyzed with four individual enzymes and eight different enzyme combination systems. The degree of hydrolysis and the distribution of amino acids in the hydrolysates were investigated. The results of this study could provide a more effective and precise method for protein hydrolysis in potato pulp.

3.2 Materials and Methods

3.2.1 Materials

Potato pulp (PP) with a 74% protein content on a dry weight basis was obtained from AVEBE B.A. (Veendam, The Netherlands). Enzyme, Alcalase 2.4 L (ALC), Flavourzyme (FLA), and Novo Pro-D (NPD) with a specific activity of 415 U^{cas}/ml, 1000 LAPU/g, and 400 U^{cas}/ml for ALC, FLA, and NPD, respectively were obtained from Novo Nordisk (Bagsværd, Denmark) and Corolase LAP (COR) with a specific activity of 350 LAPU/g from Röhm Enzyme (Darmstadt, Germany).

3.2.2 Potato pulp hydrolysate

The hydrolyzation process to obtain PP hydrolysate was carried out with a Biostat B controller unit (B.Braun, Melsungen, Germany) in a 4 l batch reactor with 7 % (w/v) substrate protein and twelve different enzyme combinations (Table 3.1). Potato pulp was dissolved in

tap water with 300 rpm stirring and was heated up to 80 °C for two hours. After cooling down to 50 °C the pH of the mixture was adjusted to 9. Sequential hydrolysis steps with various endoprotease and exopeptidase systems were conducted. Each sample was predigested with endoprotease. After two hour incubation exopeptidase was added. The next 24 hours no pH control was carried out. After the hydrolysis step the enzymes were denaturated by heat treatment at 85 °C for 15 min. After cooling down the hydrolysates were centrifuged with an Eppendorf 5415 D from Eppendorf (Hamburg, Germany) at 5900 x g for 10 min. The supernatants were collected and freezed at –20 °C for further analysis. Each of the hydrolyzation processes was performed five times. The sample analysis outcome (DH and free amino acid composition) was presented as an average value.

Table 3.1 Combination of enzyme systems for potato pulp hydrolysis (w/w)

System	Endoprotease		Exopeptidase	
	Alcalase	**Novo Pro-D**	**Flavourzyme**	**Corolase**
S1	7 %	-	-	-
S2	-	7 %	-	-
S3	-	-	7 %	-
S4	-	-	-	7 %
S5	2 %	-	5 %	-
S6	2 %	-	-	5 %
S7	-	2 %	5 %	-
S8	-	2 %	-	5 %
S9	3 %	-	4 %	-
S10	3 %	-	-	4 %
S11	-	3 %	4 %	-
S12	-	3 %	-	4 %

3.2.3 Amino acid analysis

The σ-phthalaldehyde (OPA) pre-column derivatization method was used to determine the α-amino groups in the hydrolysates (Blau and Halket, 1993). Proteins in hydrolysate were precipitated by adding 0.4 ml methanol (HPLC grade) to 0.1 ml hydrolysate and storing at 4 °C overnight. The mixture was centrifuged at 5900 x g for 15 min. The supernatant was filtrated by 0.22 µm filter and diluted 1:1 with borate buffer pH 8. The following reaction with a hydrophobic derivatizing agent (OPA) yielded a product detectable for chromatography. A Resolve 5 µm C_{18} (4 x 150 mm) column from Waters (Eschborn, Germany) was used for

amino acids analysis. Elution was achieved by a linear gradient elution (Table 3.2) formed from eluent A [0.05 M Na_2HPO_4 pH 7:CH_3OH:THF (96:2:2)] and eluent B [CH_3OH: H_2O (65:35)]. The flow rate was kept constant at 1 ml/min with a temperature of 30 °C. Fluorescence detection was performed with a Shimadzu RF-10AXL fluorescence detector (Exciation 330 nm (band pass filter), emission 420 nm (cut-off filter)).

Table 3.2 Gradient elution program used for the σ-phthalaldehyde pre-column derivatization method

Time (min)	Eluent A (%)	Eluent B (%)
0	100	0
50	0	100
55	0	100
60	100	0
67	100	0

The total number of α-amino groups in PP was determined by acid hydrolysis. 1 g (dry weight) of PP was hydrolyzed with 100 ml 6 N HCL by reflux under O_2-free nitrogen at 110 °C for 24 h. The number of free α-amino groups in the PP was assayed by HPLC OPA pre-column derivatization method as described above for the enzymatic hydrolysates.

3.2.4 Degree of Hydrolysis (DH)

DH indicates the extend of the reaction. This is the ratio of released α-amino acids in the hydrolysates to the total α-amino acids obtained after acid hydrolysis of the PP.

$$\% \text{ DH} = \frac{h}{h_{tot}} \times 100\ \%$$

3.3 Results and Discussion

3.3.1 Enzymatic hydrolysis of Potato Pulp

Many industrial enzyme reactions are not run at a fixed pH value, but slowly drift from a starting pH to a terminal pH (Novo Nordisk). Thus, many processes do not have adequate control, and the pH may fluctuate around the required pH value (Doucet et al., 2003; Linarés et al., 2000). A key parameter for monitoring the protein hydrolysis reaction is the degree of hydrolysis (DH). In order to reduce the operation costs and to used a simple process design, the hydrolyzation processes were conducted without pH control.

The DH of the PP hydrolysates produced by four different protease enzymes (S1-S4 in

Table 3.1) after 26 hours are shown in Fig. 3.1. The S3 (7 % FLA (w/w)) curve illustrates the course of PP protein hydrolysis: the DH increases rapidly in the first hour after the enzyme was added and slows down after ten hours. The initial rate of S1 (7 % ALC (w/w)) is only the half of S3 and stable after 30 min. In S2 (7 % NPD (w/w)) and S4 (7 % COR (w/w)) hydrolyzation rates are very low and stable. The total DH are 8 %, 3 %, 22 %, and 2 % for S1, S2, S3, and S4, respectively.

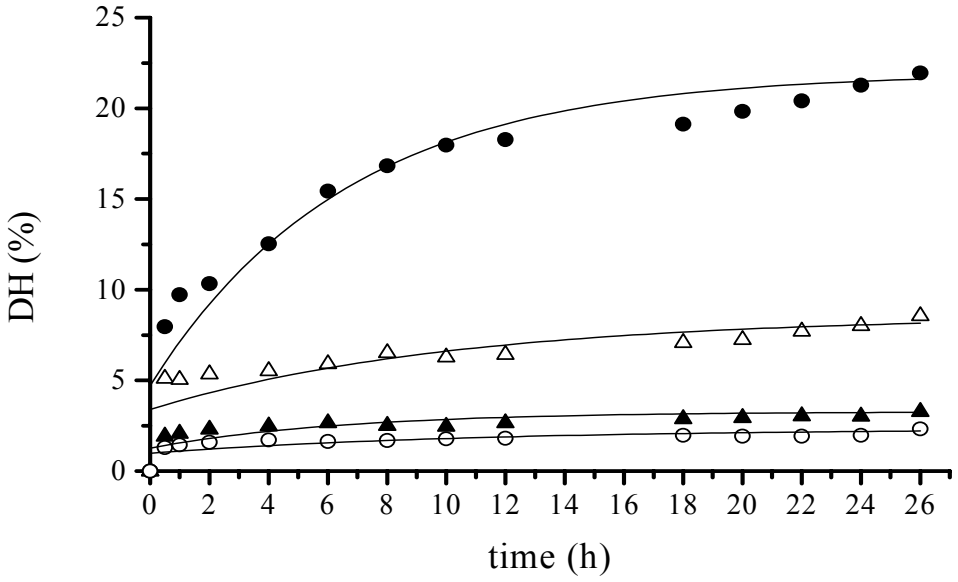


Fig. 3.1 Hydrolyzation curve of potato pulp protein with 4 different individual protease enzymes in 4 l reactor, 300 rpm stirring at 50 °C, without pH control

—△— S1 (7 % ALC), —▲— S2 (7 % NPD),
—●— S3 (7 % FLA), —○— S4 (7 % COR)

The main reason for the highest DH by FLA in comparison to the others three enzymes is the FLA composition. This enzyme is produced by fermentation of selected strains of *Aspergillus oryzae* and contains both endoprotease and exopeptidase activities (Hrčková et al., 2002; Pedroche et al., 2002), whereas the others show endo- or exopeptidase activities only. Although ALC is an endoprotease like NPD it presents the higher activity since this enzyme is a serine alkaline protease produced by a selected strain of *Bacillus licheniformis* and has broad specificity of peptide bonds hydrolyzation (Doucet et al., 2003). Sukan and Andrews have observed a higher DH for ALC to hydrolyze casine and skim milk powder, too, when compared with other enzymes such as pepsin and chymotrypsin. They presumed that this enzyme contains several different proteinases each with different specificities (Sukan and Andrews, 1982).

To obtain a hydrolysate with DH greater than 22 % it is necessary to use more than one protease. Generally, one type of enzyme in a one-step reaction cannot achieve such a high DH in a reasonable period of time (Pedroche et al., 2002). Thus, different combination of enzyme systems were examined. After adding endoprotease, the pH value of the reaction mixtuers decreased slowly from the initial pH 8.5 to the pH around 7, which is the optimum pH value for exopeptidase. After 24 h of exopeptidase hydrolysis the final pH value dropped to around 6.5. Therefore, the enzyme should be active in the given pH interval. Predigestion with endoproteases (ALC or NPD) increases the number of terminal sites, facilitating the hydrolysis by the exopeptidases (FLA or COR). With this sequential hydrolysis hydrolysates with higher DH were generated after 24 hours of hydrolyzation time (Fig. 3.2 and Fig. 3.3).

In the combination of enzyme systems a low initial hydrolyzation rate of both endo-proteases was found during the first hour of the predigestion processes (Fig. 3.2 and Fig. 3.3). Using a high initial substrate concentration, the enzyme catalyzed reaction was decreased by excess substrate and insufficient enzyme concentration effects (Moreno and Cuadrado, 1993). After an exopeptidase like FLA was added the hydrolyzation rate of PP increased rapidly during the initial stage whereas it was lower and stable in the first hour after COR adding. The highest DH, 44 %, was obtained with the enzyme mixture S5 (2 % ALC + 5 % FLA (w/w)), and the lowest, 17 %, with the two enzyme S8 (2 % NPD + 5 % COR (w/w)) and S12 (3 % NPD + 4 % COR (w/w)) systems.

In the following experiments the endoprotease concentration was increased from 2 to 3 %, while exopeptidase concentration was decreased from 5 % to 4 %. Using higher ALC concentration, the DH was slightly increased to approximately 10 % and 8 % when combined with FLA and COR, respectively (Fig. 3.2), but was stable when NPD was used (Fig. 3.3), since NPD is a specific endoprotease, whereas ALC shown a broad specificity and has a higher activity than NPD (Fig. 3.1) as mentioned above. However, the low DH of both enzymes originated from the excess substrate and insufficient enzyme concentration effects, and the detection method, as well. The reaction of the OPA derivatization method with peptides produced about 10 to 20 times less fluoresence than the reaction with amino acids (Méndez et al., 1985). Endoproteases catalyzed the peptide bonds cleavage in the protein molecules and liberated more small peptides than free amino acids (Aaslyng, 1998). Therefore, the DH resulting from this detection method were low, although from the results of MALDI a high amount of low concentrated small peptides can be found in the hydrolysates (results not shown here).

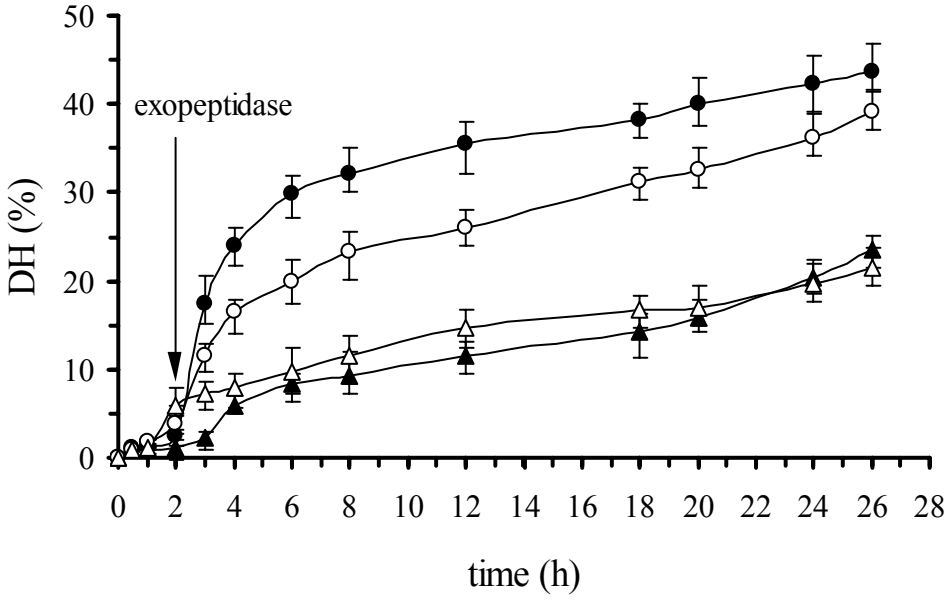


Fig. 3.2 Hydrolyzation curve of potato pulp protein with the combination of endoprotease ALC and exopeptides FLA or COR in 4 l reactor, 300 rpm stirring at 50 °C, without pH control

—●— S5 (2 % ALC + 5 % FLA), —○— S9 (3 % ALC + 4 % FLA),
—▲— S6 (2 % ALC + 5 % COR), —△— S10 (3 % ALC + 4 % COR)

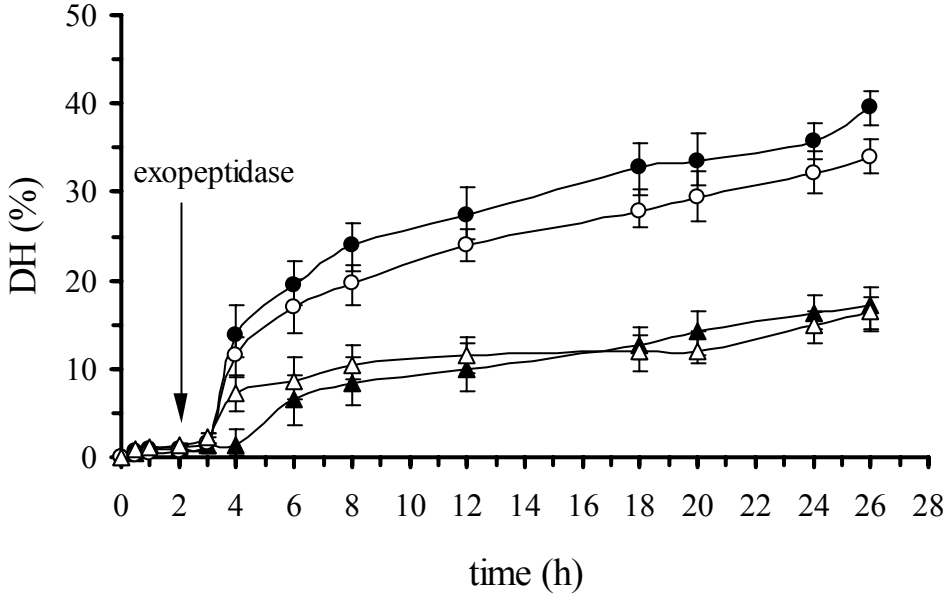


Fig. 3.3 Hydrolyzation curve of potato pulp protein with the combination of endoprotease NPD and exopeptides FLA or COR in 4 l reactor, 300 rpm stirring at 50 °C, without pH control

—●— S7 (2 % NPD + 5 % FLA), —○— S11 (3 % NPD + 4 % FLA),
—▲— S8 (2 % NPD + 5 % COR), —△— S12 (3 % NPD + 4 % COR)

In addition, the effect of exopeptidase concentration on the overall hydrolyzation processes was investigated. The concentration of the both exopeptidases (FLA and COR) was decreased from 5 % to 4 %. The same pattern of DH curves, but lower DH were obtained when using the lower amount of exopeptidase. In the case of lower FLA concentration the DH decreased approximately to 9 % in S9 (3 % ALC + 4 % FLA (w/w)) (Fig. 3.2) and to 15 % in S11 (3 % NPD + 4 % FLA (w/w)) (Fig. 3.3) compared to the S5 (2 % ALC + 5 % FLA (w/w)) and S7 (2 % NPD + 5 % FLA (w/w)). Furthermore, the combination of ALC + COR performed a higher DH than the combination of NPD + COR (approximately 27 % and 23 %) when the concentration of COR decreased from 5 % to 4 %, respectively. Therefore, the more effective enzymes used in PP hydrolyzation are AlC and FLA, and the combination of ALC and FLA is suitable not only for hydrolyzing PP protein, but for other protein types with high DHs, as well (Aaslyng et al., 1999; Vioque et al., 1999). The S5 (2 % ALC + 5 % FLA (w/w)) enzyme system seemed to be most efficient particularly in a rapid hydrolysis of PP proteins and it generates the highest DH. The total DH are 44 %, 40 %, 39 %, and 34 % for enzyme combination S5, S7, S9, and S11, respectively.

3.3.2 Amino acid distribution in potato pulp hydrolysates

Before any polypeptides or proteins solution can be subjected to an amino acid analysis, the sample has to be hydrolyzed to break down the peptide bonds between the amino acids. The amino acid distribution of PP before and after enzymatic hydrolysis is shown in Table 3.3 and Table 3.4. The difference of the amino acids distribution between native potato pulp (NPP) and hydrolysates treated with pure proteases and a combination of protease enzyme systems was significant. Generally, the NPP lacked of aromatic amino acids (His, Phe, Trp, and Tyr) and a sulfur-containing amino acid methionine (Met). These amino acids increased significantly in the case of FLA and ALC hydrolysates due to the combination of endo- and exoprotease activities of FLA and the broad specificity of ALC which preferentially hydrolyzed peptide bonds containing aromatic amino acids residues (Ward, 1983). In the case of enzyme combinations (Table 3.4) the total amount of free amino acid (Σ faa) increased significantly when using the combination of ALC + FLA and NPD + FLA, while the lowest Σ faa was generated by the combination of NPD + COR. In comparison to S7 (2 % NPD + 5 % FLA (w/w)) and S11 (3 % NPD + 4 % FLA (w/w)) only 43 % and 47 % of Σ faa was produced from S8 (2 % NPD + 5 % COR (w/w)) and S12 (3 % NPD + 4 % COR (w/w)), respectively. The free amino acid Leu was the highest produced (around 18 % of Σ faa) for each enzyme combination system. The amino acid distribution and the Σ faa were illustrated in Table 3.4.

Σ faa content in the 2 % ALC + 5 % FLA (w/w) hydrolysate strongly increased in the first four hours of hydrolysis after 5 % FLA was added, and further increase for the next 14 h at a lower rate (Table 3.5). This means the exopeptidase activity started as soon as the substrate was present. After 2 h the total amount of free Gly (3.13 mg/g protein) and Trp (2.22 mg/g protrein) were produced in the hydrolyzation process (around 37 % of the final amount of these amino acids) but there was no production of Arg and Met during this peroid. The amount of these amino acids increased to around 64 % and 48 % in the third and to around 96 % and 69 % in the sixth hour compared to the final amount in the twenty-sixth hour, for Arg and Met, respectively. The Σ faa added up to 40 %, 55 % and 68 % of the final amount after three, four, and six hours, respectively.

Table 3.3 Effect of enzymatic hydrolysis on the free amino acids distribution (mg/g protein) of potato pulp in the presence of different protease enzymes

Amino Acids (mg/g)	**Native Potato Pulp**	**Hydrolysate hydrolyzed with**			
		Alcalase	**Novo Pro-D**	**Flavourzyme**	**Corolase**
Essential					
His	-	3,75	0,01	3,22	1,93
IIe	0,12	0,92	0,66	12,65	0,30
Leu	0,28	5,51	0,98	26,99	0,65
Lys	0,23	2,62	0,31	6,86	2,54
Met	-	1,06	0,40	6,54	0,01
Phe	-	2,84	1,18	14,83	0,20
Thr	0,06	3,87	1,14	9,34	0,84
Trp	-	1,27	0,70	2,20	0,06
Val	0,08	1,26	0,59	14,14	0,31
Non-essential					
Ala	0,01	2,94	0,02	8,63	0,10
Arq	-	0,59	0,09	0,05	0,08
Asp	0,06	1,28	0,81	6,30	0,61
Asn	0,01	1,11	0,89	3,50	0,26
Glu	0,03	3,83	1,01	6,83	0,51
Gln	0,03	0,04	2,05	6,86	0,00
Gly	1,22	10,48	4,76	4,96	3,17
Ser	0,73	5,96	3,32	3,68	2,11
Tyr	0,02	2,03	0,73	8,60	0,18
Total	**2,88**	**51,36**	**19,65**	**146,18**	**13,86**
DH (%)	**-**	**8,56**	**3,28**	**21,95**	**2,33**

It is a tremendous advantage of enzymatic hydrolyzation process that Asn, Gln, and other sensitive amino acids are not destroyed during the process, and that it does not induce a racemization, whereas chemical digestion destroys and racemises them (Fountoulakis and Lahm, 1998). 86 % of the final Gln amount was produced in the third hour. The amounts of Asx (Asp and Asn) and Glx (Glu and Gln) were slightly decreased from the eighteenth hour to the twentieth hour and then slightly increased until the end of the hydrolyzation process (26 h). The amount of Asp and Glu increased between the twentieth and twenty-sixth hour of the hydrolysis, but this increase was mainly a result of deamination of Asn and Gln. The amounts of His, Met, and Trp were constant, and the other amino acids increased from the twentieth hour to the twenty-sixth hour, except Arg, Asn, and Gln which slightly decreased, because the long time hydrolysis has a negative effect on the concentration of these amino acids. Only the first 4 hours of FLA hydrolysis seemed to be an important period for amino acid production. The final Σ faa was 306.11 mg/g protein, and the final DH was 44 %.

3.4 Conclusion

The endoprotease ALC and exopeptidase FLA suit best for hydrolyzing the PP protein whereas the endoprotease NPD and exopeptidase COR present a very low hydrolyzation activity for this protein type. The total DH are 8 %, 22 %, 3 %, and 2 % for ALC, FLA, NPD, and COR, respectively. To improve the hydrolyzation process the combination between an endoprotease, ALC or NPD, with FLA is more efficient than using the individual protease solely. A 44 % DH was obtained from the combination between 2 % ALC + 5 % FLA (w/w). The amount of FLA influences the DH and the obtained end products: 3 % ALC + 4 % FLA (w/w) generates 39 % DH, 2 % NPD + 5 % FLA (w/w) generates 40 % DH, and 3 % NPD + 4 % FLA (w/w) generates 34 % DH. This follows from the fact that FLA is a mixture between endo- and exoprotease.

Enzymatic hydrolysis can be used to improve the quality of PP proteins and thus increase their value since it occurs under mild condition (Doucet et al., 2003). In this study the amino acids concentration in the enzymatic hydrolysates is significantly higher than in the NPP, especially aromatic amino acids (His, Phe, Trp, and Tyr) and sulfur-containing amino acid Met. For some of amino acids, especially Asn and Gln, the continued hydrolysis even had a negative effect, since they were deaminated. For many free amino acids the first four hours after adding FLA seem to be the most important period. From the twentieth hour of hydrolysis only a low increase in the Σ faa was observed.

Table 3.4 Effect of enzymatic hydrolysis on the free amino acids distribution (mg/g protein) of potato pulp in the presence of different protease combination systems

Amino Acids (mg/g)	Native Potato Pulp	Alcalase				Novo Pro-D			
		2%		3%		2%		3%	
		5% FLA	5% COR	4% FLA	4% COR	5% FLA	5% COR	4% FLA	4% COR
Essential									
His	-	8.28	7,00	6,94	6,37	7,42	4,94	6,31	3,88
Iie	0,12	20.74	15,24	20,43	13,93	21,84	11,54	17,19	10,29
Leu	0,28	46.33	30,08	49,52	28,26	50,26	21,84	42,02	19,88
Lys	0,23	20.13	12,77	11,43	10,92	11,54	9,48	25,12	9,16
Met	-	10.7	6,59	7,93	6,14	8,24	3,71	8,70	3,36
Phe	-	26.16	16,48	26,74	15,73	26,78	11,54	21,85	11,03
Thr	0,06	19.12	15,24	17,23	12,18	16,89	9,89	15,23	6,00
Trp	-	6.19	5,36	6,69	4,96	7,00	4,12	5,77	4,03
Val	0,08	27	17,30	23,77	15,66	24,72	13,18	20,01	11,25
Non-essential									
Ala	0,01	14.23	4,94	12,94	4,52	13,18	5,36	10,87	3,86
Arq	-	14.72	2,47	14,44	1,85	9,48	0,41	3,06	0,79
Asp	0,06	11.17	0,41	5,27	0,65	5,77	0,41	4,32	0,31
Asn	0,01	13.98	4,12	13,16	4,52	13,18	3,30	10,19	2,51
Glu	0,03	14.87	3,30	8,44	3,72	9,06	0,41	7,97	0,54
Gln	0,03	5.44	1,24	10,94	0,52	10,71	2,88	6,11	2,49
Gly	1,22	8.25	4,53	5,99	4,39	6,59	2,47	6,43	11,01
Ser	0,73	16.32	4,94	12,99	4,71	13,18	4,53	10,13	3,46
Tyr	0,02	22.48	14,01	21,90	12,55	22,25	9,48	15,21	7,75
Total	**2,88**	**306.11**	**165,62**	**276,72**	**151,58**	**277,69**	**119,48**	**236,45**	**111,6**
DH (%)		**43.75**	**23,44**	**39,23**	**21,55**	**39,60**	**17,10**	**33,91**	**16,53**

Table 3.5 Free amino acids distribution (mg/g protein) of potato pulp protein hydrolysate depending on a hydrolyzation time with 2 % Alcalase + 5 % Flavourzyme

Amino Acid (mg/g)	**2 % Alcalase**			**5 % FLA**								
	0.5 h	**1 h**	**2 h**	**3 h**	**4 h**	**6 h**	**8 h**	**12 h**	**18 h**	**20 h**	**24 h**	**26 h**
Essential												
His	-	-	0,62	3,26	4,38	4,78	6,13	7,13	8,32	7,99	8,11	8,28
Ile	0,48	0,43	0,46	6,37	9	12,09	12,62	15,54	16,82	18,62	19,59	20,74
Leu	0,73	0,49	0,43	22,36	28,09	35,56	35,11	39,69	41,11	43,99	45,24	46,33
Lys	0,67	2,74	3,66	9,93	12,88	12,57	15,16	13,72	8,1	18,39	17,43	20,13
Met	0,02	0,01	-	5,1	6,07	7,33	7,92	8,97	10,04	9,84	10,28	10,7
Phe	0,45	0,12	0,35	8,05	14,67	16,43	18,74	21,79	22,74	24,54	25,4	26,16
Thr	0,31	0,29	0,95	7,05	9,11	12,87	9,57	15,2	14,61	17,59	18,76	19,12
Trp	-	0,07	2,22	1,31	3,04	4	4,29	4,78	5,88	5,45	6,02	6,19
Val	0,4	0,39	0,2	9,87	12,57	16,17	17,81	21,45	23,32	25,22	26,39	27
Non-essential												
Ala	0,35	0,52	0,69	5,5	7,15	9,56	9,53	11,23	12,44	13,3	13,9	14,23
Arg	-	-	-	9,49	11,97	14,09	15,13	15,99	19,33	16,18	15,78	14,72
Asp	0,15	0,3	0,38	2,24	3,28	6,18	5,41	6,99	6,81	9,02	10,5	11,17
Asn	0,08	0,06	0,08	5,33	9,13	7,74	11,4	12,86	23,96	14,48	14,05	13,98
Glu	0,29	0,28	0,3	3,75	5,62	9,44	8,44	10,62	13,06	13,48	14,32	14,87
Gln	0,22	0,08	0,08	4,7	5,49	5,24	6,27	6,37	6,81	5,86	5,76	5,44
Gly	1,26	1,78	3,13	4,18	5,34	6,26	8,03	6,93	7,09	7,59	7,96	8,25
Ser	0,61	0,96	1,9	6,53	8,33	12,07	11,23	13,1	14,29	15,32	15,76	16,32
Tyr	0,45	0,15	0,95	6,1	12,35	15,16	15,18	17,11	12,68	19,85	20,12	22,48
Total	**6,47**	**8,67**	**16,4**	**121,12**	**168,47**	**207,54**	**217,97**	**249,47**	**267,41**	**286,71**	**295,37**	**306,11**
DH (%)	**1,08**	**1,46**	**2,56**	**17,45**	**23,88**	**29,85**	**31,11**	**35,55**	**38,19**	**40,95**	**42,25**	**43,75**

CHAPTER 4 INFLUENCE OF COLUMN TYPES AND CHROMATOGRAPHIC CONDITIONS ON THE RP-HPLC PEPTIDE SEPARATION OF THE ENZYMATIC POTATO PULP HYDROLYSATE

4.1 Introduction

In peptide chemistry HPLC has gained an important role as a technique for separation, purification and the study of peptides due to its sensitivity, speed, and resolving power (Grego et al., 1986). Existing HPLC methods are ion-exchange, size-exclusion chromatography and RP-HPLC. However, RP-HPLC predominates and is often selected for separating peptides derived from chemical or enzymatic hydrolysis of proteins (Hearn, 1991). Proteins and peptides are made up of chains of amino acids, each with a side group attached. Some of these are hydrophobic, i.e. the amino acids alanine, valine, leucine, and isoleucine have hydrocarbon chains attached, proline has a cyclic hydrocarbon attached, and phenylalanine, tyrosine, and tryptophan have aromatic rings attached. Proteins arrange themselves in aqueous solutions to achieve a minimum of free energy by hiding most of their hydrophobic groups inside the molecule and leaving the charged groups outside. Some hydrophobic groups will remain on the outside, exposing hydrophobic regions that are available for association with hydrophobic groups on the matrix of the stationary phase. RP-HPLC is most effective with small proteins or peptides. Proteins with a large number of hydrophobic residues tend to become denatured and may bind tightly with the stationary phase thus it may be difficult to elute it from the column (Wheelwright, 1991).

The separation mechanism of this method depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and an immobilized hydrophobic alkyl chain ligand on the stationary phase (Jaroniec, 1993). These hydrophobic groups are responsible for the specific chromatographic character of the column. The separation is based mainly on two different factors: (i) the strength of the hydrophobic interactions of each component in the sample with the hydrophobic surface of the column matrix, and (ii) the elution strength of the organic solvent in the mobile phase (Zhou et al., 1990). The least hydrophobic molecules elute first, followed by molecules of increasing hydrophobicity. Therefore, molecules that have some degree of hydrophobic character, such as peptides and proteins, can be separated with excellent recovery and resolution (Scopes, 1994).

Selectivity in separation processes is affected by several factors, including the characteristics of the columns and the chromatographic conditions. The column matrix is

made of silica gel supports modified by alkylation where the chain length of the alkyl substituent varies in length between methyl and C_{18} (octadecyl). The most popular lengths are n-butyl (C_4), n-octyl (C_8), and n-octadecyl (C_{18}) (Skrzydlewska et al., 1998). Katz et al. also reported the influence of chromatographic conditions such as column temperature and mobile phase composition on separation effectivity (Katz et al., 1998). Various factors influence the way a particular peptide interacts with the column, especially the characteristics of the peptide itself, i.e. amino acids composition (Zhou et al., 1990), residues sequence (Houghten and De Graw, 1987), peptide length (Mant et al., 1989), and the presence of any secondary structure (Steer et al., 1998). The RP-HPLC of peptides and proteins with varying temperature has also allowed an insight into the role of conformation in these substances' retention behavior (Chen et al., 2003). The usage of elevated temperatures for the RP-HPLC separation of peptides or proteins has been advocated, primarily as a mean of increasing column efficiency or decreasing running time (Antia and Horváth, 1988). A few studies have shown that a change in column temperature can also affect the separation selectivity of such samples (McKern and Edskes, 1993).

Studying the mobile phase's influence on the retention in RP-HPLC is important for understanding the regularities of the retention and the separation mechanisms of the substances in the chromatographic process. Binary eluents consisting of a weak and a strong solvent are widely used. The major constituent of the mixed eluent is a high polar solvent, e.g. water, whereas a less polar solvent, e.g. acetonitrile, methanol, and isopropanol etc., is used as an organic modifier to control the process of elution. Dilute trifluoroacetic acid (TFA 0.1 % v/v) is often used in the mobile phase because it is a good solvent for peptides and proteins and does not absorb the far UV (205–220 nm), whereas the peptide bond absorbs it. Moreover the incorporation of 0.1 % TFA in the mobile phase system acidifies the mobile phase and also acts as an ion pair type reagent to enhance the hydrophobic nature of peptides by neutralizing charges. Another advantage of TFA is it volatility and easy removability (Zapala, 2003). Elution is usually carried out by using increasing gradient of an organic solvent concentration. The organic component must possess sufficient polarity to keep the peptides in the solution, and sufficient non-polarity to elute the peptides from the matrix. The use of stronger non-polar organic solvents such as acetonitrile in the mobile phase will reduce retention time. The optimum solvent composition has to be obtained by trial and error. In this study, the effect of various silica matrices of the column, as well as chromatographic conditions as temperature and mobile phase composition were investigated.

4.2 Materials and Methods

4.2.1 Chemicals. Acetronitrile (ACN) and Isopropanol (IPA) HPLC-grade were obtained from Carl Roth GmbH (Karlsruhe, Germany) and trifluoroacetic acid (TFA) from Fluka Chemie GmbH (Taufkirchen, Germany).

4.2.2 Potato pulp hydrolysate. The hydrolyzation process to gain PP hydrolysate was carried out with 7 % substrate protein content. PP (AVEBE B.A., Veendam, The Netherlands) was dissolved in tap water with 300 rpm stirring and heated at 80 °C for two hours. After cooling down to 50 °C the solution was adjusted to pH 9. Sequential hydrolysis steps with endo- and exoprotease were conducted. Each sample was predigested with 2 % ALC (Novo Nordisk, Bagsværd, Denmark). After two hours of incubation, 5 % FLA (Novo Nordisk, Bagsværd, Denmark) was added. For the next 24 hours pH was not controlled. After the hydrolysis the enzymes were deactivated by heat treatment of the hydrolysates at 85 °C for 15 min. The hydrolysates cooled down and were centrifuged at 5900 x g for ten minutes. The supernatants were collected and freezed at –20 °C for further analysis.

4.2.3 Equipment. The HPLC was a Merck Hitachi LaChrom system equipped with a D-7000 interface, a L-7100 pump, a L-7350 column oven, a L-7200 auto sampler, a L-7612 solvent degasser, and a L-7455 diode array detector (Merck, Darmstadt, Germany).

4.2.4 Methods. The PP hydrolysates resulting from a 2 % ALC + 5 % FLA enzymatic hydrolyzation were separated by RP-HPLC on four different column types (Table 4.1). The combination of an ACN gradient (Fig. 4.1) and an IPA gradient (Fig. 4.2) at three different temperatures, i.e. 30, 40 and 50 °C, were utilized. The chromatograms were detected by the diode array detector at wavelengths of 214, 220 and 280 nm.

Table 4.1 Characteristics of the different RP-HPLC columns

No.	Type	Brand name (manufacture)	Particle Size (nm)	Shape	Surface Area (m^2/g)	Pore Size (°A)	Carbon Loading (%)	Endcapped (Y/N)
1	RP-C_4	Nucleosil C_4 (Macherey-Nagel)	5	S	200	120	5	N
2	RP-C_8	Nucleosil C_8 (Supleco Inc.)	5	S	350	100	9	N
3	RP-C_{12}	Jupiter Proteo C_{12} (Phenomenex)	4	S	475	90	15.0	Y
4	RP-C_{18}	Nucleosil C_{18} (Macherey-Nagel)	5	S	350	100	15.0	Y

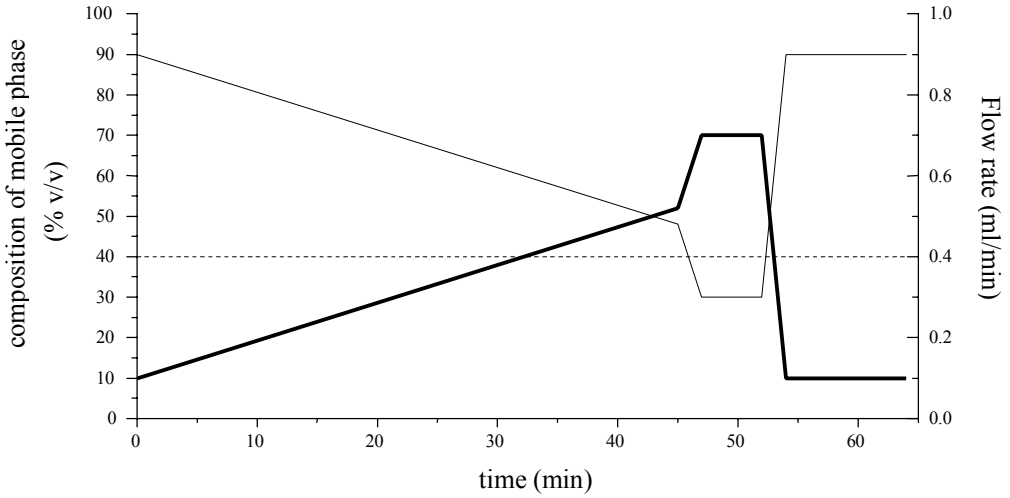


Fig. 4.1 Acetonitrile gradient elution profile for peptides separation of PP hydrolysates,

——— eluent A: 0.1% TFA in water

——— eluent B: 0.1% TFA in acetonitrile

····· flow rate

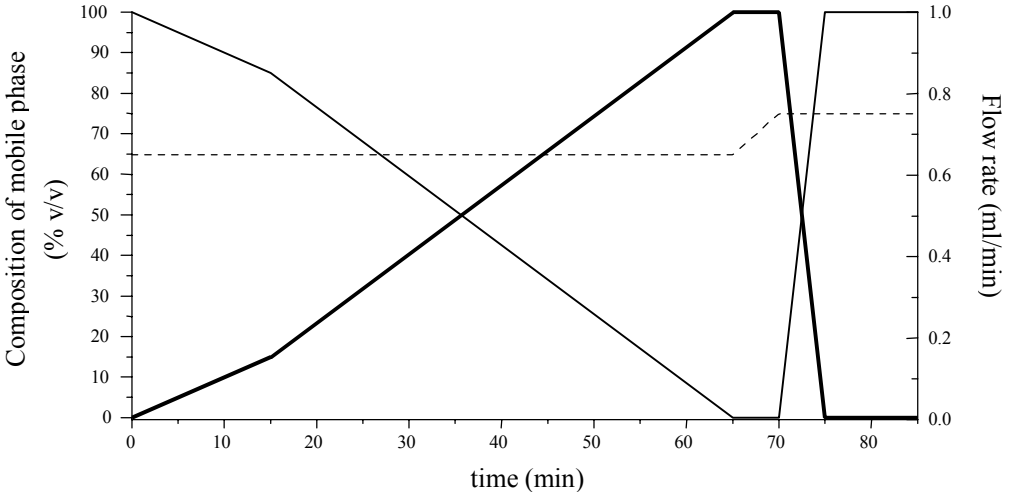


Fig. 4.2 Isopropanol gradient elution profile for peptides separation of PP hydrolysates,

——— eluent A: 1% acetonitrile in water

——— eluent B: 0.1% TFA in 38 % isopropanol

····· flow rate

4.3 Results and discussion

4.3.1 Effect of the column stationary phase

When selecting a column for a given separation many factors must be considered, which include the general suitability for the type of sample and purpose of the separation, sufficient efficiency and resolution (support particle size, shape and porosity, and its ability to bind a sufficient concentration of suitable active species), the chemical and mechanical stability of the support and the stationary phase itself under the particular separation conditions, and reasonable speed and cost of the separation. It is well known that silica-based supports represent the majority of stationary phase materials in RP-HPLC columns and that they contain covalently bound alkyl chains of different lengths (Akapo and Simpson, 1994). The most common alkyl chains are C_4, C_8, and C_{18} (Skrzydlewska et al., 1998). In this study the separation chromatograms of PP hydrolysates on Nucleosil C_4, Nucleosil C_8, Jupiter Proteo $C_{12,}$ and Nucleosil C_{18} were obtained under identical chromatographic conditions. Little difference in selectivity for peptides was observed by alkyl chain length variation (Fig. 4.3). The separation on a C_4 column resulted in a poor resolution, while C_{12} (n-dodecyl) and C_{18} were found to be the most effective columns for the peptides separation, because the separation on RP-column often correlates with their increasing relative hydrophobicities (characteristic of RP-HPLC conditions are hydrophobic stationary phase, non-polar eluting mobile phase). The short alkyl chain allows the separation of large peptides and proteins with high efficiency and symmetrical peaks, whereas long alkyl chains are better suited for separation of weaker hydrophobic character or smaller peptides (Scott and Simpson, 1992).

Although the C_{18} column is recommended for the separation of enzymatic hydrolysis products and for small hydrophilic peptides (2-10 residues) (Bradshaw, 1998), it lacks a resolution between the first two peaks in the chromatogram of this column. In difference to that the C_{12} column shows such a resolution. Moreover the peak height of each peak obtained from C_{12} was significantly higher than C_{18} (Fig 4.3) due to the physical properties of the column. C_{12} has smaller particle and pore sizes than C_{18} (Table 4.1). The separation efficiency of the columns is defined by column peak capacity (P) rather than by theoretical plates. P represents the number of peaks that can be separated within gradient time. It depends on the length and particle size of the stationary phase. Namely, the separation efficiency is directly linked to the particle size. The maximum performance occurs at a shorter analysis times if the particle size is decreased (Neue, 1997). Hence, the most efficient column for the peptides separation of PP hydrolysates is the Jupiter Proteo C_{12} column.

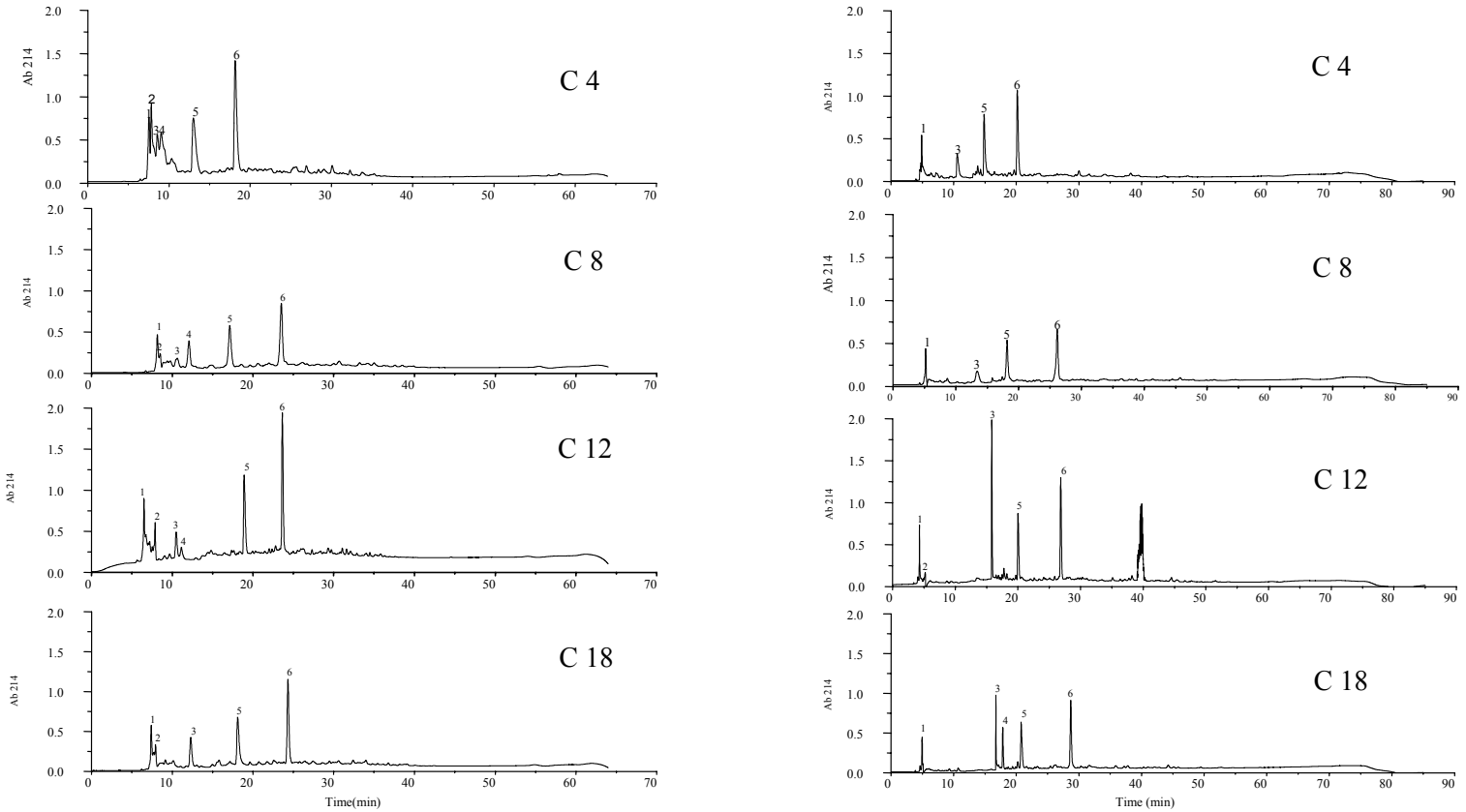


Fig. 4.3 Peptide separation of PP hydrolysates on various columns eluted with ACN gradient (left), and IPA gradient (right) at 30 °C, detected at 214 nm.

4.3.2 Effect of the chromatographic conditions

4.3.2.1 Influence of the mobile phase

Solvent strength and solvent type are widely recognized as important variables in RP-HPLC separation (Snyder et al., 1997). Hence, studying the influence of the mobile phase on the retention in HPLC is important for understanding the regularities of the retention and mechanisms of substance separation in chromatographic processes. The influences of various mobile phase compositions on column efficiency were investigated in this study. Normally, water, ACN, methanol, and IPA are often used as a mobile phase (Sadek, 2002). The hydrophobic strength of these organic solvents decreases in the order of isopropanol > acetonitrile >> methanol (Hartman et al., 1986). Although ACN was commonly used as mobile phase, alcohol, particularly IPA is occasionally used to improve the solubility of hydrophobic peptides. The comparison of the separation chromatograms by ACN and IPA gradients with various stationary phase are shown in Fig. 4.4 and 4.5.

The resolution between peak 1 and peak 2 (hydrophilic peptides) from C_8 and C_{18} column lacks when IPA gradient was substituted by ACN gradient, since during gradient development not only the percentage of the organic eluant but also the pH and ionic strength change. Thus, differences in the pH of the initial mobile phase can lead to significant changes in the elution properties of some peptides. Hydrophilic peptides, that elute during the first part of the gradient (the more polar), are the most affected (Wilson et al., 1981). On the other hand the IPA gradient elution of the hydrophobic peptides (elute later from the column) was improved, especially by C_{12} column. This is due to the fact that IPA is a stronger RP-solvent (less polar solvent) than ACN. It will distribute to a greater extent into the stationary phase, thus the separation of hydrophobic peptides was improved (Sugihara et al., 1981).

4.3.2.2 Influence of the column temperature

Not only the choice of the mobile phase but also the temperature has an effect on the separation of substances by HPLC. The temperature affects solvent viscosity, column back pressure and retention time. The chromatograms obtained from different column temperatures are shown in Figure 4.4 and 4.5. With the C_8 column both the resolution and the asymmetry were not significantly different at a different point of temperature. Similar results are obtained with the C_{12} and C_{18} columns, as well. Although the increase in column temperature results in large column plate numbers and lower mobile phase viscosity an insignificant resolution between peak 3 and 4 was achieved. A co-elution of these peaks was obtained from C_{12} column eluted with the ACN gradient at 40 and 50 °C. Similar phenomena were apparent in the profile of C_{18} column eluted with the IPA gradient. In contrast the resolution from C_{12}

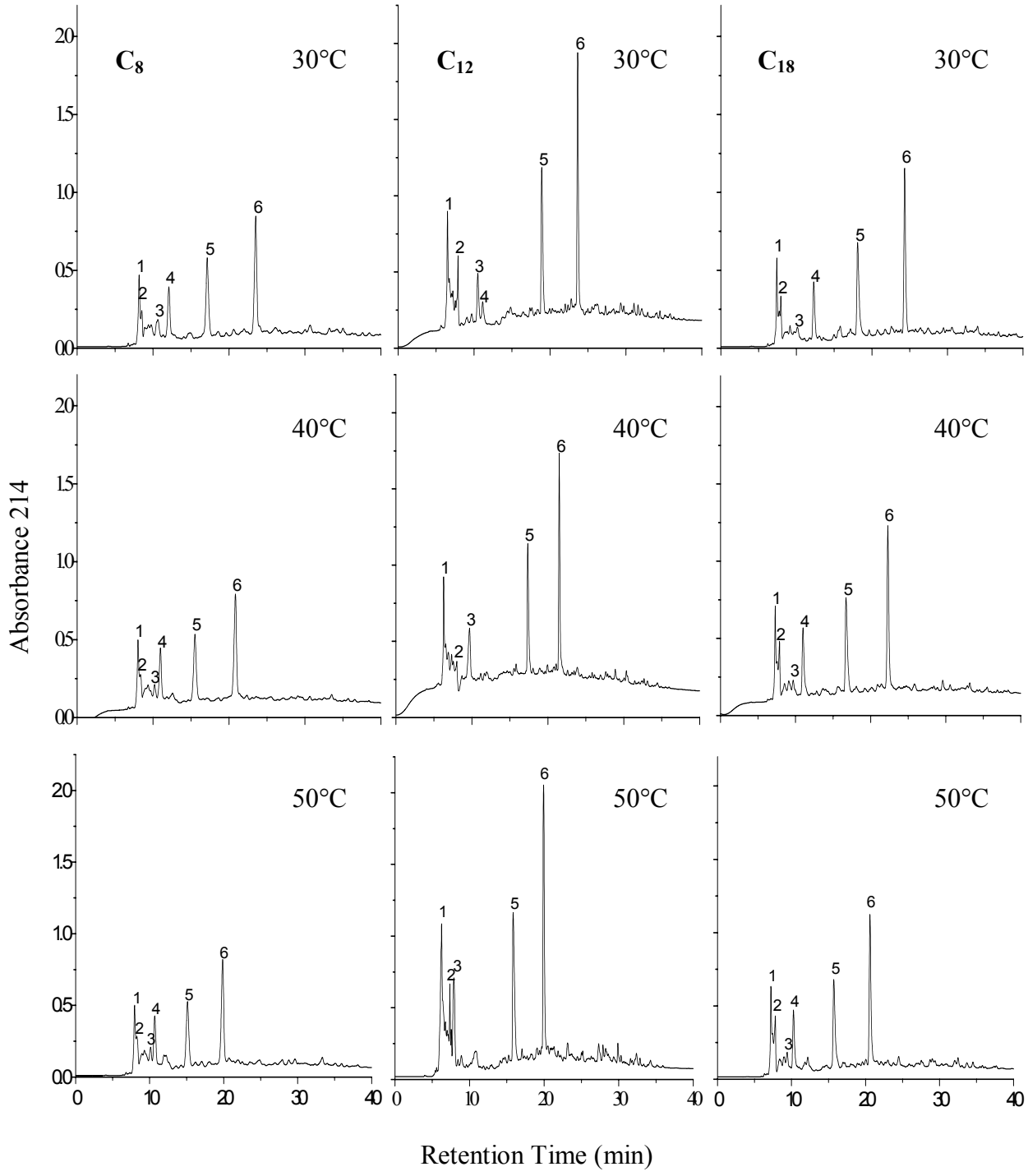


Fig. 4.4 Effect of temperature and stationary phase on peptides in PP hydrolysates separation by RP-HPLC eluted with acetonitrile gradient

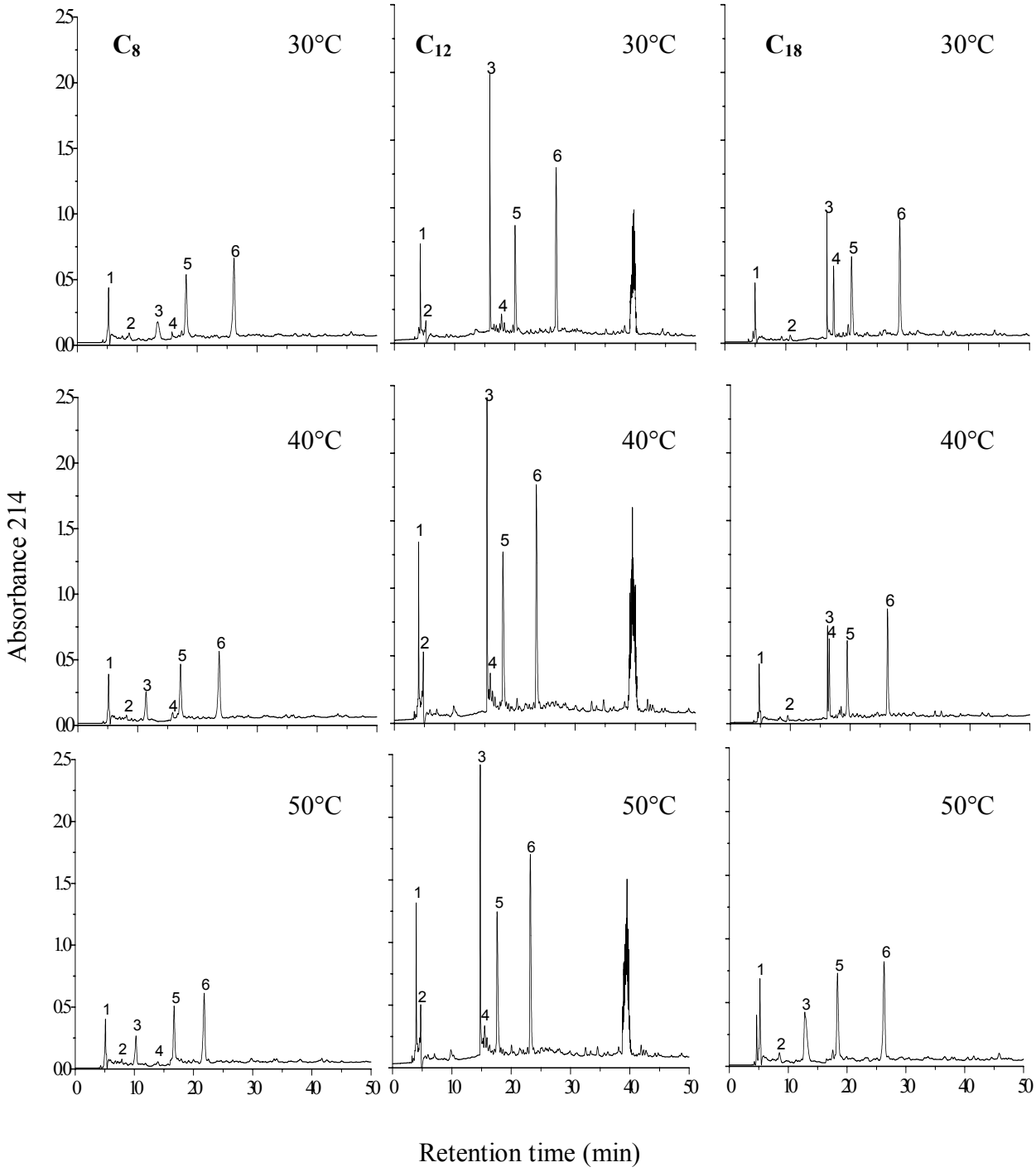


Fig. 4.5 Effect of temperature and stationary phase on peptides in PP hydrolysates separation by RP-HPLC eluted with iso-propanol gradient

eluted with IPA and from C_{18} eluted with ACN improved when the temperature increased.

Tables 4.2 and 4.3 summarize the changes in retention time and peak width with the temperature for each peak. It can be deserved that the retention time and peak width decrease as temperature increase from 30 to 50 °C. Increasing temperature generally results in increasing the capacity factors, in increasing the mass transfer between the mobile phase and stationary phase (Lee et al., 2003; Li and Carr, 1997), and in changing the chemical nature of the solute (Knox, 1973). This might be explained by the decreasing solvent viscosity and increasing solute solubility in the mobile phase (Dolan, 2002). Therefore, the most effective chromatographic condition for the separation of the peptides in the PP hydrolysate is an ACN gradient at 30 °C.

4.4 Conclusion

Because of the complexity of the hydrolysates to be separated, very high resolution is required. Thus the problem of the separation is what techniques or selective separation should be used. A chromatographic method is a setting condition of the separation which controls the run of a chromatographic analysis. A great deal of attention has been focused on the method development, optimization, and validation. The aim of the method development and optimization is to find the best separation conditions. The most common strategy for method development in practice is still trial and error combined with experimental knowledge and practical rules. The chromatographic separation depends on several factors, including physical and chemical properties of the hydrolysates and of the stationary and mobile phases. The criterion for method development is the maximization of the minimum separation (or resolution) between adjacent peaks. The most important reason for an insignificant resolution is the slowness of mass transfer and of the sorption/desorption processes. Those can be accelerated by choosing stationary particle types that permit rapid solute transport. In this study the alkyl chain length and particle and pore sizes of the stationary phase affected the separation efficiency (resolution) but did not affect the selectivity. Thus, similar elution selectivity chromatograms with different in resolution were obtained from different stationary phases. Short alkyl chain resulted in a poor resolution while long alkyl chain gave a superior resolution.

Not only the stationary phase, but also the chromatographic conditions, e.g. the type of the mobile phase and temperature have a powerful influence on the separation. The different selectivity profiles between ACN and IPA gradient elutions were observed. The resolutions of hydrophilic (peaks 1 and 2) and hydrophobic (peaks 3 and 4) peptides were improved when eluted with ACN gradient and IPA gradient, respectively. The retention time and peak width

decreased as temperature increased. Increasing temperature resulted in the co-elution of hydrophobic peptides (peaks 3 and 4) when eluted with ACN and IPA.

Nowadays more than 600 kinds of RP columns are commercially available worldwide and newer chemical and physical properties of them are brought into the market. Thus, a further study can test the other columns with different chemical (as cyano-, propyl-, diphenyl-bonded phase, and polymer based etc.) and physical properties (as smaller particle and pore size, shorter column length etc.) to improve the selectivity and resolution. At the same time the chromatographic conditions should be developd to obtain a better separation profile of the PP hydrolysate peptides.

Table 4.2 Summary of retention times and peak widths for peptide separation of potato pulp hydrolysate with acetonitrile gradient as a function of temperature and column stationary phases

	Nucleosil C_4			**Nucleosil C_8**			**Jupiter Proteo C_{12}**			**Nucleosil C_{18}**		
	30 °C	40 °C	50 °C	30 °C	40 °C	50 °C	30 °C	40 °C	50 °C	30 °C	40 °C	50 °C
Peak 1	7.54[a] (0.41)[b]	-	7.43 (0.41)	8.17 (0.36)	8.02 (0.36)	7.97 (0.31)	6.50 (0.22)	6.28 (0.20)	6.25 (0.16)	7.40 (0.21)	7.29 (0.22)	7.22 (0.19)
Peak 2	7.83 (0.28)	-	7.68 (0.22)	8.52 (0.34)	8.34 (0.36)	8.26 (0.33)	7.88 (0.19)	8.01 (0.23)	7.36 (0.19)	7.94 (0.19)	7.85 (0.22)	7.81 (0.16)
Peak 3	8.57 (0.37)	-	7.99 (0.31)	10.63 (0.72)	10.19 (0.50)	10.13 (0.45)	10.49 (0.46)	9.69 (0.59)	7.89 (0.44)	12.29 (0.55)	10.99 (0.41)	10.26 (0.41)
Peak 4	9.05 (0.52)	-	8.60 (0.59)	12.08 (0.72)	10.97 (0.54)	10.69 (0.57)	11.13 (0.73)	-	-	15.81 (0.41)	-	12.20 (0.30)
Peak 5	13.01 (1.08)	-	11.59 (0.63)	17.13 (0.79)	15.51 (0.77)	15.11 (0.77)	18.91 (0.46)	17.35 (0.46)	15.85 (0.44)	18.11 (0.51)	16.72 (0.46)	15.71 (0.44)
Peak 6	18.15 (0.74)	-	15.02 (0.61)	23.52 (0.66)	20.85 (0.67)	19.87 (0.64)	23.65 (0.34)	21.50 (0.28)	19.93 (0.42)	24.33 (0.58)	22.29 (0.52)	20.60 (0.51)

[a] Retention time (min).
[b] Peak width (min).

Table 4.3 Summary of retention times and peak widths for peptide separation of potato pulp hydrolysate with iso-propanol gradient as a function of temperature and column stationary phases

	Nucleosil C_4			Nucleosil C_8			Jupiter Proteo C_{12}			Nucleosil C_{18}		
	30 °C	40 °C	50 °C	30 °C	40 °C	50 °C	30 °C	40 °C	50 °C	30 °C	40 °C	50 °C
Peak 1	4.90[a] (0.16)[b]	-	4.83 (0.15)	5.20 (0.26)	5.14 (0.23)	5.11 (0.23)	4.29 (0.19)	4.02 (0.14)	3.94 (0.16)	4.97 (0.19)	4.81 (0.17)	4.64 (0.23)
Peak 2	10.58 (0.61)	-	7.75 (0.61)	8.65 (0.50)	8.14 (0.41)	7.89 (0.41)	5.22 (0.16)	4.79 (0.14)	4.75 (0.15)	10.83 (040)	9.57 (0.35)	8.49 (0.35)
Peak 3	13.85 (0.28)	-	-	13.41 (1.03)	11.40 (0.71)	10.27 (0.74)	15.83 (0.18)	15.37 (0.16)	14.79 (0.15)	16.75 (0.11)	16.21 (0.11)	12.82 (1.2)
Peak 4	14.86 (0.56)	-	13.20 (0.41)	16.95 (0.48)	15.85 (0.50)	13.97 (0.48)	17.77 (0.26)	16.70 (0.25)	15.92 (0.22)	17,85 (0.28)	16.49 (0.22)	-
Peak 5	20.17 (0.56)	-	16.70 (0.35)	18.16 (0.51)	17.17 (0.47)	16.72 (0.48)	20.02 (0.40)	18.01 (0.44)	17.62 (0.40)	20.79 (0.47)	19.49 (0.43)	18.37 (0.42)
Peak 6	-	-	-	26.15 (0.92)	23.63 (0.84)	21.81 (0.73)	26.84 (0.35)	23.56 (0.47)	23.21 (0.55)	28.71 (0.50)	26.23 (0.49)	26.29 (0.43)

[a] Retention time (min).
[b] Peak width (min).

CHAPTER 5 SEPARATION OF POTATO PULP HYDROLYSATE PEPTIDES

5.1 Introduction

The separation and purification of peptides in protein hydrolysates have been a major focus of biological and analytical chemists for many years. In general, separation procedures lead to either a preparative level in order to isolate one or more individual components from a mixture for further investigations, or an analytical level with the aim of identifying an amount of some or all mixture components. The physicochemistry properties of the peptides have a clearly influence upon the separation method. Peptides are fractionated from each other on the basis of differences in physicochemical characteristics such as charge, hydrophobicity surface, size, and solubility (John et al., 2004). An additional factor that significantly influences the separation method is the purpose of which peptides are separated. If this is for research application, separation to homogeneity is usually the most important goal that requires multiple steps, duration of method, costs, and percentage yields of a final product. If it is separated for a commercial application economic as well as technical factors require a minimal level of separation (Walsh, 2002). Therefore, the initial step in any separation method is designated to (i) liberate and concentrate the peptides of interest, and (ii) to remove contaminants.

Since column chromatography is applied to the protein and peptide separation, it refers to the separation of different peptide types from each other according to their different partitioning between two phases: a solid stationary phase (the chromatographic beads, usually packed into the cylindrical column), and a mobile phase (usually buffer or organic solvent). Most separation protocols require more than one step to achieve the desired level of product purity. Size exclusion chromatography (SEC) and RP-HPLC are attractive procedures for separating peptides in protein hydrolysates (Visser et al., 1992; Pellerin et al., 1985). Ordinarily, SEC has been used for fractioning the protein hydrolysates, often as a first step prior to RP-HPLC (Lemieux and Amiot, 1990).

SEC or gel filtration chromatography is a widely used and well documented method to analyze the molecular weight of peptides from the protein hydrolysates. It continues to be an efficient separation method in peptide purification and presents the most simple separation mechanism in chromatography (Barth et al., 1994). Molecules are actually separated according to the basis of different shape and molecular size as they pass through a packing medium in the column. Accordingly, the smallest molecules in a sample migrate into the

smallest pores, while the bigger molecular weight molecules prevent them from penetrating into the pores. Therefore the largest molecules will elute first and the smallest molecule will elute later.

RP-HPLC is a powerful tool for the separation of peptides from protein hydrolysate. The separation is based on the differences in hydrophobicity. The case of small peptides (less than 15 residues) retention time depends only on the amino acid composition, while the retention time of large peptides is influenced by other effects, e.g. peptide length (Mant et al., 1988). Nevertheless, there is a limitation in the sensitive and/or selective detection after a chromatographic run, especially the short peptides with less than 10 amino acids often lack a suitable chromophore or fluorophore and have to be detected by UV-wavelength range from 205 to 230 nm (Antonis et al., 1994). This range is quite unspecific since a lot of substances show UV-absorption therein. Thus, solving this problem of low in sensitivity and selectivity, the derivatization procedure offers a lot of possibilities for peptide detection. Peptides have at least two functional groups where the derivatization might take place: the primary amino group at the N-terminus (except the peptides which contain a proline, acylated amino acid residue or pyroglutamate at the N-terminus) and the carboxyl group at the C-terminus. The amino group is an important at all in peptide derivatization methods in contrary to the carboxylic group. Since the carboxylic group has to be activated itself before it can react with amines, lots of activated acids and other reagents are available for the derivatization of peptides and amino acids at amino groups (Koller and Eckert, 1997). The use of precolumn derivatization of amino acids with σ-phthalaldehyde in the presence of 2-mercaptoethanol and their subsequent separation by RP-HPLC provides an excellent method for the amino acid analysis and determination of peptides (Stegehuis et al., 1991; Méndez et al., 1985; Jones et al., 1981; Joys and Kim, 1979).

The primary objective of this work was to develop a method for the multi-dimensional chromatographic separation of peptides from the PP hydrolysates that would ensure the chromatographic purity of the obtained fractions. For this purpose, the peptides in PP hydrolysates were separated to different molecular size fractions by Superdex Peptide HR10/30 column (SEC method) with various mobile phases. All fractions obtained from SEC were further separated by RP-HPLC. The σ-phthalaldehyde precolumn derivatization and non-derivatization methods were compared. The molecular weight and peptide sequence of the resulting peptide fractions will be identified in a subsequent experiment.

5.2 Materials and Methods

5.2.1 Chemicals

Acetronitrile(ACN) HPLC-grade was obtained from Carl Roth GmbH (Karlsruhe, Germany). Sodium dihydrogen phosphate anhydrous, Sodium chloride, and Trifluoroacetic acid (TFA) from Fluka Chemie GmbH (Taufkirchen, Germany). Amino acids and small peptides standard: Asn, Trp, Gly-Leu, Gly-Gly-Gly, Gly-Gly-Leu, Phe-Gly-Gly-Phe, Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe), HPLC peptides standard mixture, and amino acids standard mixture from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany).

5.2.2 Equipment

- *Size exclusion chromatography (SEC):* The Techlab LCP 4001 pump equipped with Triathlon auto-sampler and Sama S-3702 UV-Vis detector.
- *Reverse-phase chromatography (RP-HPLC)*: Two HPLC systems were employed in the studies. One for the derivatization method comprised the Techlab LCP 4001 pump, the Triathlon auto-sampler and the Shimadzu RF-10AXL fluorescence detector. The other system for the non-derivatization method consisted of a Merck- Hitachi LaChrom system equipped with a D-7000 interface, a L-7100 pump, a L-7350 column oven, a L-7200 auto sampler, a L-7612 solvent degasser, and a L-7455 diode array detector (Merck, Darmstadt, Germany).

5.2.3 Methods

- *SEC:* The PP hydrolysates resulting from the 2 % ALC + 5 % FLA (w/w) enzymatic hydrolysis were separated by SEC on a Superdex Peptide HR10/30 column (Pharmacia Biotech, Uppsala, Sweden) at room temperature. The PP hydrolysates were filtraterd through a 0.22 μm filter. The injection volumes amounted to 20 μl and 50 μl. The elution buffers were 0.25 M NaCl in 0.02 M phosphate buffer pH7.2 (PBS), 0.05 %, 0.1 %, and 0.15 % TFA, and 15 %, 20 %, and 30 % ACN. To each ACN eluent 0.1 % (v/v) TFA was added. Each elution buffer was run with isocratic elution at a flow rate of 0.5 ml/min for 60 minutes. Eluate was monitored at 214 nm. The peaks of the hydrolysate were isolated and pooled separately. All eluate fractions were concentrated by freeze drying for further investigations.
- *RP-HPLC:* All concentrated SEC eluate fractions were further separated by two separate RP-HPLC methods. One is the σ-phthalaldehyde (OPA) pre-column derivatization method. The eluate fractions were diluted 1:1 with borate buffer pH 8. A Resolve 5 μm C_{18} (4 x 150 mm) column was used and eluted with gradient elution. Eluent A was 0.05 M Na_2HPO_4 pH 7:CH_3OH:THF (96:2:2) and eluent B was CH_3OH:H_2O (65:35). The gradient elution was 0-55 min 100-0 % eluent A, 55-60 min 0-100 % eluent A, and 60-67 min 100 % eluent A at a flow rate 1 ml/min, and a temperature of 30 °C. Fluorescence detection

was performed with a Shimadzu RF-10AXL fluorescence detector (Exciation 330 nm (band pass filter), emission 420 nm (cut-off filter)). The peaks were isolated and pooled separately. The eluate was concentrated by freeze drying for a further investigation. The other RP-HPLC method was carried on the Jupiter Proteo C_{12} (90 °A, 4 μm, 250 X 4.6 mm) column (Phenomenex, Aschaffenburg, Germany). The ACN gradient at the temperatures 30 °C was utilized. Eluent A was Mili-Q water and eluent B was ACN. 0.1 % (v/v) TFA was added to both eluents. The gradient is illustrated in Fig 5.1. The chromatograms were detected with the diode array detector at a wavelength of 214 nm. The peaks were isolated and pooled separately. The eluate was concentrated by freeze drying for further investigation.

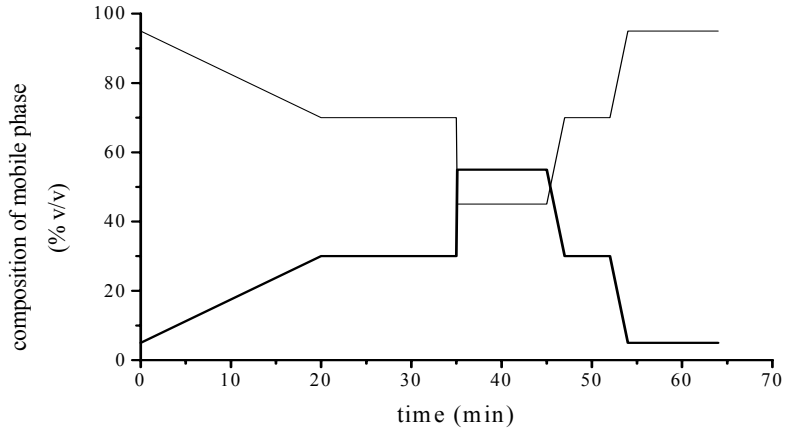


Fig 5.1 Gradient elution profile of non-derivatization RP-HPLC method

eluent A: 0.1 % TFA in water
eluent B: 0.1 % TFA in acetonitrile

5.3 Results and Discussion

5.3.1 SEC Separations

SEC is an attractive and convenient method to investigate peptide profiles in protein hydrolysates if the molecular size or approximate molecular mass distribution therein has to be determined (Makarov and Szpunar, 1998). The accuracy depends on the calibration curve constructed for a series of known molecular mass standards. In this study this technique was employed for fractionated the peptides in PP hydrolysates by using a Superdex Peptide HR10/30 column. This column packing material consists of dextran covalently bonded to highly cross-linked porous agarose. The optimum fractionation range is between 100 and 7000 Da. The chromatographic behavior of standard amino acids and peptides (detail of the standards used are given in Table 5.1) on this column eluted with 20 % ACN containing 0.1 % TFA (v/v) as a mobile phase is shown in Fig. 5.2.

Table 5.1 Identification, code employed and molecular weight of amino acids and peptides standards used to characterize the Superdex Peptide HR 10/30 column

Code	Standard	Molecular weight (Da)
1	Angiotensin II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)	1046.2
2	Phe-Gly-Gly-Phe	426.5
3	Gly-Gly-Leu	245.3
4	Gly-Gly-Gly	189.2
5	Gly-Leu	188.2
61-65	amino acid standard mixture + Asn + Trp	
61	Glu	147.1
	His	155.2
	Thr	119.0
	Arg	174.2
	Ala	89.0
	Val	117.1
	Ile	131.2
	Leu	131.2
	Lys	182.6
62	Asp	133.1
	Glu	147.1
	Asn	132.1
	Ser	105.1
	Gly	75.1
	Thr	119.1
	Met	149.2
	Lys	182.6
63	Phe	165.2
64	Tyr	181.2
65	Trp	204.2
71-73	peptide standard mixture	
	Gly-Tyr	
	Val-Tyr-Val	238.2
	Methionine Enkephalin Acetate	379.5
	(Tyr-Gly-Gly-Phe-Met)	573.7
	Leucine Enkephalin Acetate	
	(Tyr-Gly-Gly-Phe-Leu)	555.6
	Angiotensin II Acetate	
	(Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)	1046.2

A non-ideal size exclusion effect was observed because the standards did not elute in a linear manner from the column in order of decreasing molecular mass. The aromatic amino acid tryptophan (Trp (65)) was strongly absorbed on this column, while tyrosine (Tyr (64)) and phenylalanine (Phe (63)) showed little interaction, eluted earlier. Moreover, standard small peptides containing Phe, e.g. Phe-Gly-Gly-Phe (2), eluted from the column after the expected retention time. Smith and FitzGerald also reported the retardation of Trp, Tyr, and di- and tripeptides containing these amino acid residues when eluted with 30 % ACN + 0.1 %

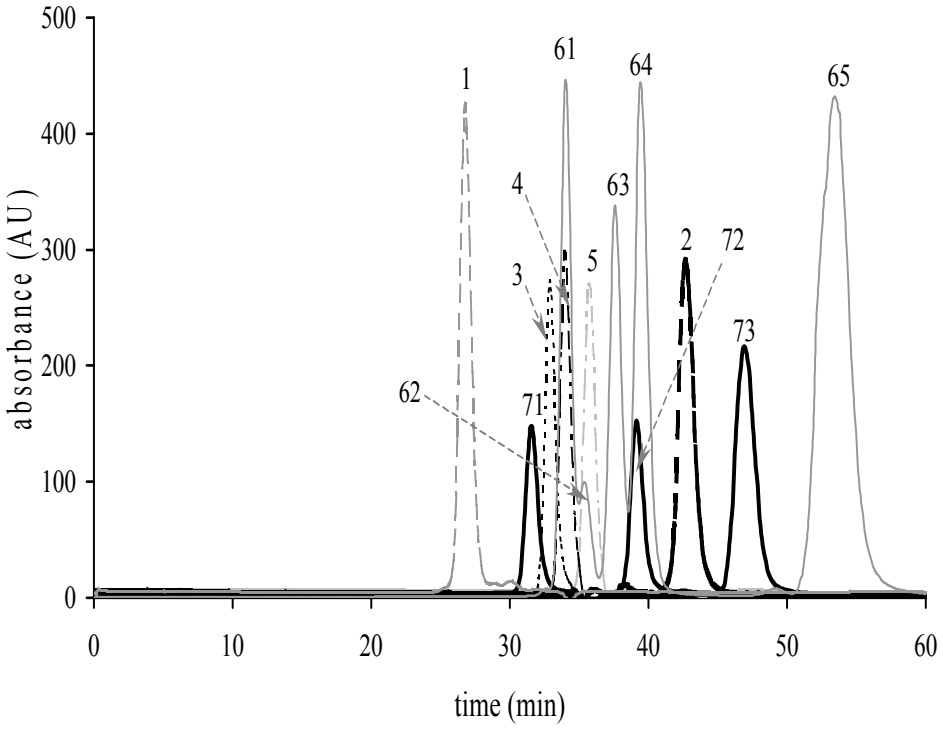


Fig 5.2 Elution profile of peptide and amino acid standards on Superdex peptide HR 10/30 column eluted with 20 % ACN + 0.1 % TFA (v/v), at the flow rate of 0.5 ml/min and detected at 214 nm

TFA (v/v) from this column (Smith and FitzGerald, 1997). This is probably due to the hydrophobic interactions between these amino acids and the column matrix. All the amino acids, Phe, Tyr, and Trp, are the most hydrophobic, with side chain hydorphobicities of 11.1, 12, and 12.5 kJ.mol^{-1}, respectively (Wallace, 1992). This corresponds to the order in which they eluted from the column, while the ideal SEC is only a steric effect that contributes to the columns' performance. The molecular size strictly determines the position at which it elutes. Furthermore, the mixed peptide standards were separated into three fractions (71 to 73). Angiotensin II in the mixed standard (71) eluted later than a pure angiotensin II (1). The co-elution of Gly-Tyr and Val-Tyr-Val, and of Met-Enkephalin Acetate and Leu-Enkephalin Acetate were obtained. This results out of the electrostatic interactions among species, especially small peptides and amino acids, in the sample, and may cause deviations from size exclusion elution (Silvestre et al., 1994).

Many factors influence a final resolution between the peaks of the SEC separation, e.g. sample size, the ratio of the sample volume to the column volume, particle size, column dimension, pore size of packing materials, elution flow rate, elution mobile phase etc. The affect of the sample size and the various elution mobile phases were investigated. The sample

size is defined by both the injection volume as well as by the concentration of the sample solution. It should be relatively small compared to the column volume, typically 1 to 5 percent. If a high resolution is obtained and the peaks are well separated, it may be possible to determine a higher maximum sample load empirically. Since the concentration of peptides in PP hydrolysate were unknown, the effect of different injection volumes on the resolution was examined. The elution profile of the PP hydrolysate is shown in Fig. 5.3. The use of a large sample volume (50 µl) can lead to a significant band broadening, resulting in loss of the resolution between peak 3, 4, and 5, while the satisfactory resolution was obtained from a lower injection volume (20 µl). The SEC column capacity does not depend on the amount of sample adsorbed, but it on the maximum amount of the sample that can be loaded onto the column without loss of resolution. This volume depends not only on the pore volume and other characteristics of the matrix, but also on the number of contaminants in the sample (Wheelwright, 1991). For this reason, using smaller volumes will help to avoid overlapping if closely spaced peaks are eluted.

Some matrices of SEC show adsorptive characteristics under certain conditions. When the matrix presents the charges, the weak electrostatics may lead to ion exchange with oppositely charged solute molecules, or, if the matrix is more hydrophobic than the mobile phase which inducing to hydrophobic interactions with solute molecules (Irvine and Shaw, 1986). Weak electrostatic interactions can usually be avoided by the addition of salt in the elution buffer, while hydrophobic interactions are avoided by the use of less salt in the elution buffer or by the inclusion of a solvent such as acetronitrile (Konishi, 1985; Richter et al., 1983). In SEC the elution mobile phase does not directly influence the resolution since the separation depends on the differences of molecular sizes. Therefore water, neutral inorganic salt solutions (e.g. sodium or potassium chloride), the buffer solution (e.g. phosphate buffer), and organic solvents are used as a mobile phase (Regnier and Gooding, 1992). In this study phosphate buffer (PBS), TFA, and ACN were used as such.

In fact the PBS can maintain a buffering capacity during the separation process, but in this study this buffer was not a suitable one. It caused many problems for a further investigation (peptide characterization) because of poor separation resolutions between peaks 2, 3, 4, and 5 (Fig. 5.4). It was hard to collect individual fractionations. Furthermore, after lyophilization process a high salt concentration in each fraction was obtained. If an excessive salt concentration (>10 mM) in the sample is achieved, it will affect the ionization of the solute molecules in the sample to gas phase and will lead to a reduced sensitivity of MALDI mass spectral analysis (Lewis et al., 2000).

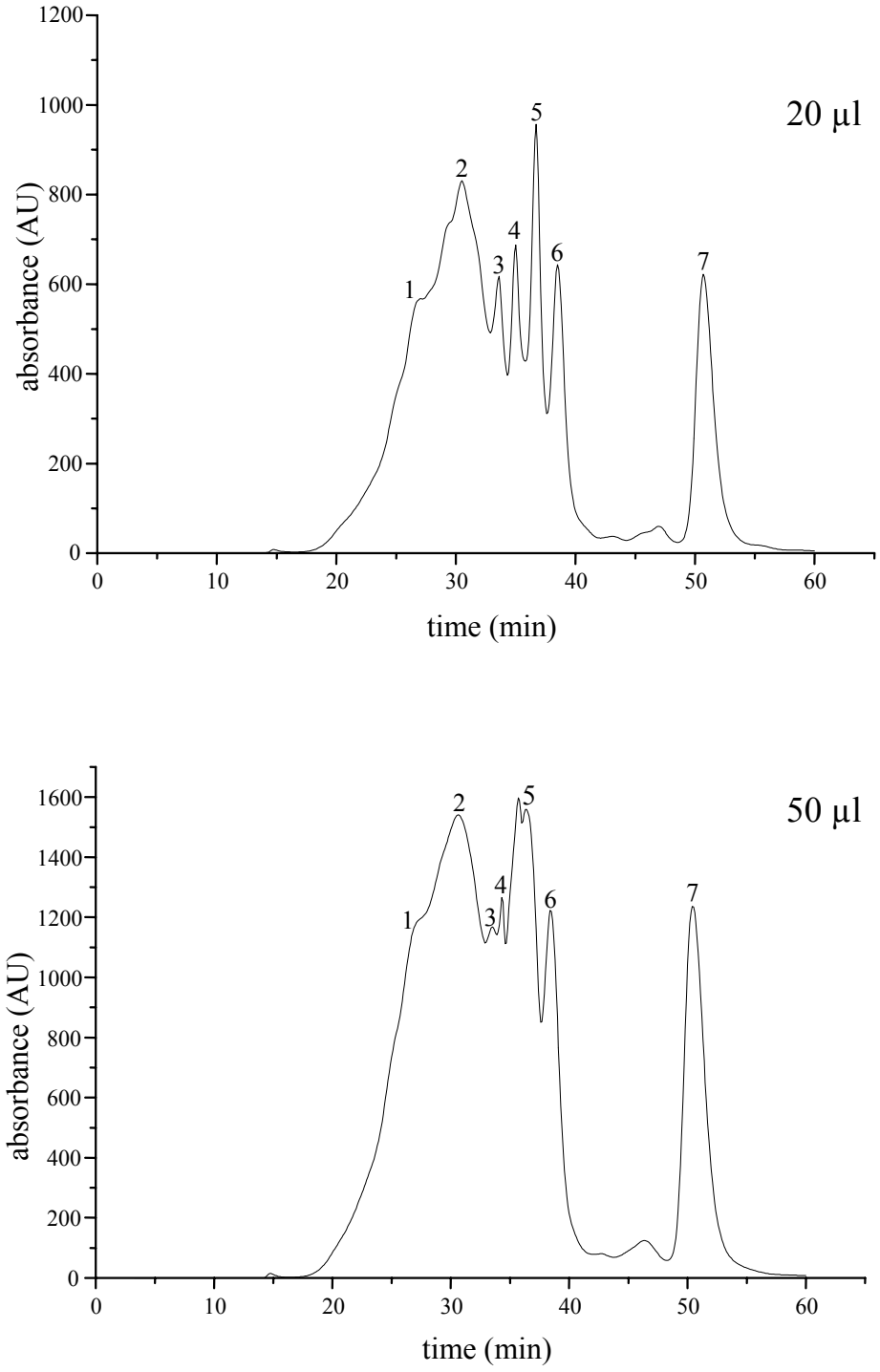


Fig. 5.3 Effect of sample volume on peptides separation of PP hydrolysates on Superdex peptide HR 10/30 eluted with 20 % ACN + 0.1 % TFA, at the flow rate of 0.5 ml/min and detected at 214 nm

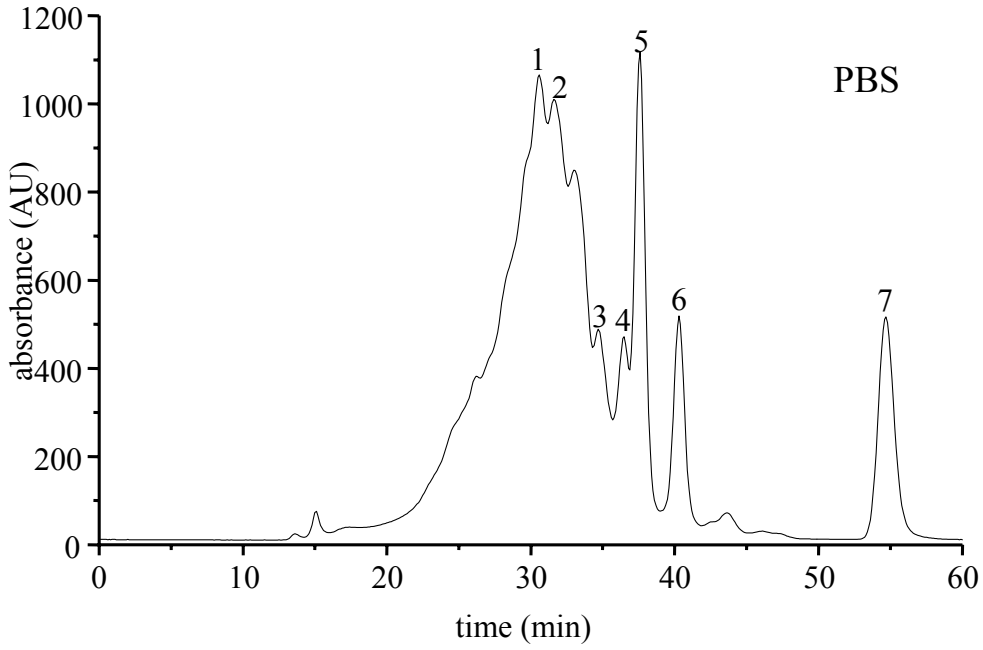


Fig. 5.4 Elution profile of potato pulp hydrolysates on Superdex peptide HR10/30 column eluted with 0.25 M NaCl in 0.02 M phosphate buffer pH 7.2, at the flow rate of 0.5 ml/min and detected at 214 nm

The Superdex peptide HR 10/30 column can be used with all eluents commonly used in SEC above pH 1 to 14. Thus, not only buffer but also TFA and ACN can be used. The elution profiles of PP hydrolysates eluted with various concentrations of TFA are shown in Fig. 5.5. Increasing the TFA concentration resulted in an increase of the retention time and better peak resolution. The poor resolutions between peaks 3, 4, and 5 were obtained when eluted with a low concentration of TFA (0.05 % TFA (v/v)), while higher TFA concentration improved the resolutions of these peaks. However, all of the solutes eluted from the column faster than from the PBS, since TFA is a strong acid and acts as ion pair agent. It associates with positively charged functional groups of peptides, suppresses the ionization of carboxyl groups, and keeps amine groups fully protonated. Thus, it eliminates any possible ion exchange interaction with basic solutes (Chen et al., 2004).

The higher organic concentration leads to a stronger mobile phase and a shorter retention time. Faster retention time and fewer peaks were observed in the chromatograms obtained from 30 % ACN, while with 15 % and 20 % ACN the PP hydrolysates were eluted from the column later and fractionated into seven fractions: S1 to S7 (Fig. 5.6). The resolution of the peak S3 to S6 eluted with 20 % ACN was superior than that eluted with 15 % ACN. No free amino acids were detected in the fraction S1 and S2. The small peptide fractions (S3-S6) were contaminated by free amino acids. The last peak in the elution profile (S7) corresponds

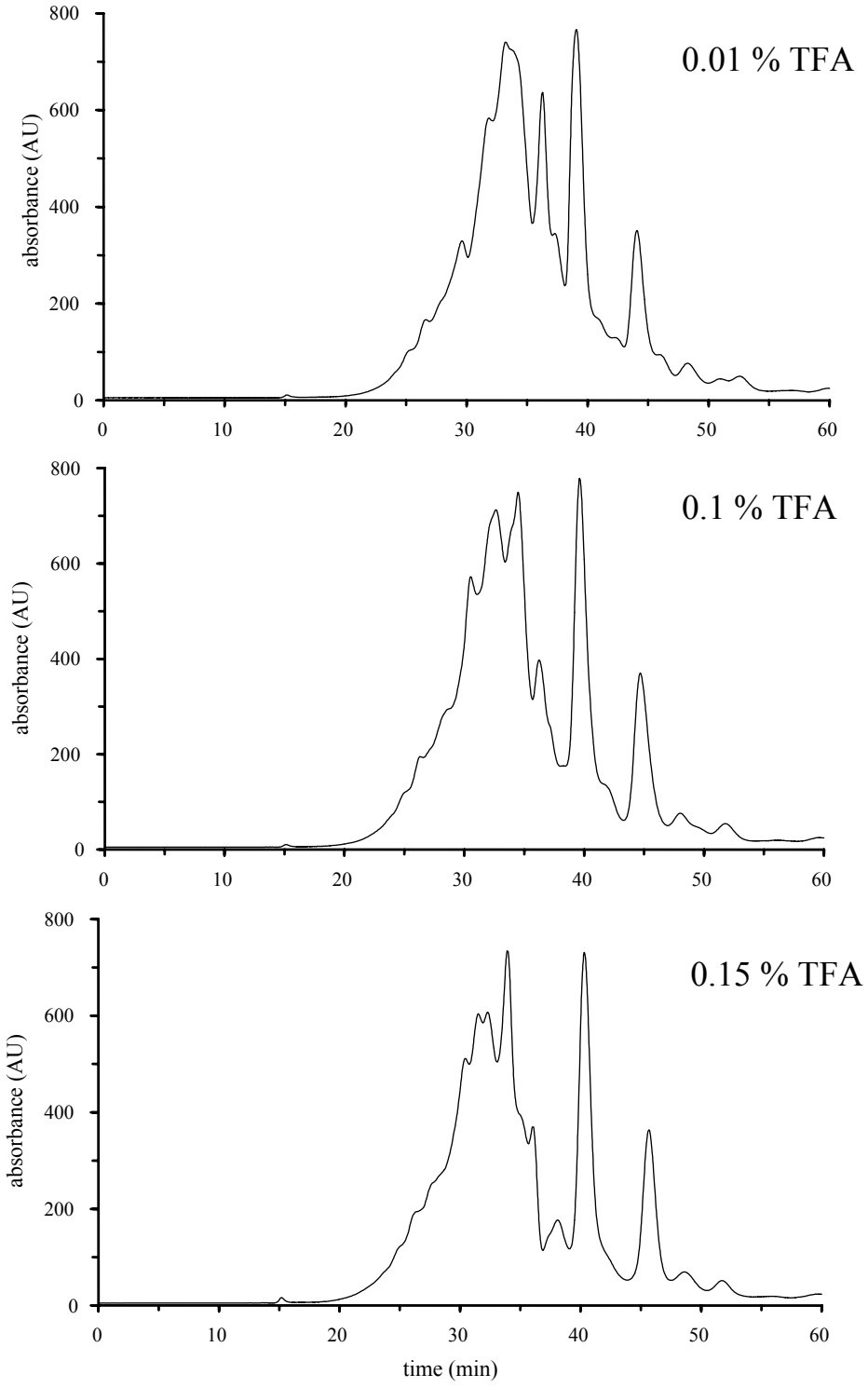


Fig. 5.5 Effect of TFA concentration on peptides separation of PP hydrolysates on Superdex peptide HR 10/30 at the flow rate of 0.5 ml/min and detected at 214 nm

to the amino acid tryptophan. This is a result of non-ideal SEC behavior of proteins and amino acids. Smyth and FitzGerald reported that the chromatographic behavior of low molecular mass peptides were effectively fractionated when eluted with high ACN concentration, but it was not suitable for proteins and free amino acids molecular mass determination (Smyth and FitzGerald, 1997). It was the biggest problem to precisely determine the largest peptides size in the hydrolysate, as the rise of the first peak was rather slow.

5.3.2 RP-HPLC separation

Fractions from Superdex Peptide HR10/30 column eluted with 20 % ACN were then run on RP-HPLC with OPA derivatization and non-derivatization methods. Fig. 5.7 illustrates the detection selectivity of each fraction obtained from SEC by OPA derivatization method. The high molecular fractions (S1 and S2) gave a poor detection signal, while the small molecular fractions (S3 to S7), which contaminated with amino acids, presented the higher signal. This is due to the reaction of the OPA in the presence of 2-mercaptoethanol with peptides, producing about 10 to 20 times less fluoresence than with amino acids (Méndez et al., 1985), but between the peaks in fractions S3 to S7, especially S4 sharpness was obtained. However, this method was not suitable for further study, since high salt concentration in each separation fraction was obtained.

Compared to the non-derivatization method (Fig. 5.8), the superior selectivity and the shorter retention time were achieved by using the ACN gradient. During the lyophilization process the S1 fraction was damaged, so no details in the respective separation profile were collected. For fractions S2 to S5 a massive amount of peaks eluted early, because of the co-elution of hydrophilic peptides. Satisfactory separations of fractions S6 to S7 were obtained. Fraction S7 mainly contains small hydrophobic peptides which eluted from the column later. Due to the hydrophobic side chains, most of the larger biologically peptides required much higher ACN concentration for elution (Meek, 1980).

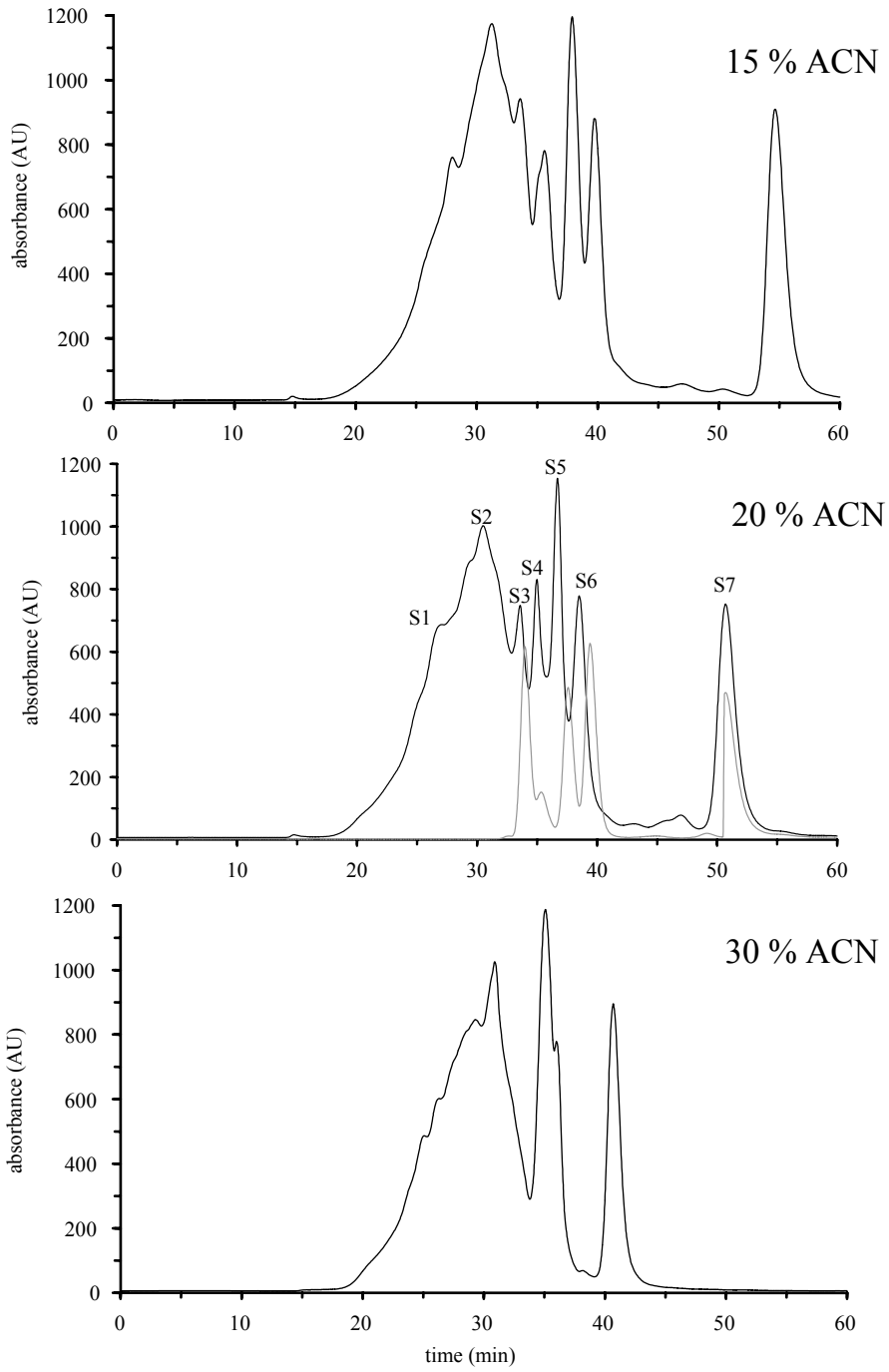


Fig. 5.6 Effect of ACN concentration on peptides separation of PP hydrolysates on Superdex peptide HR 10/30 at the flow rate of 0.5 ml/min and detected at 214 nm

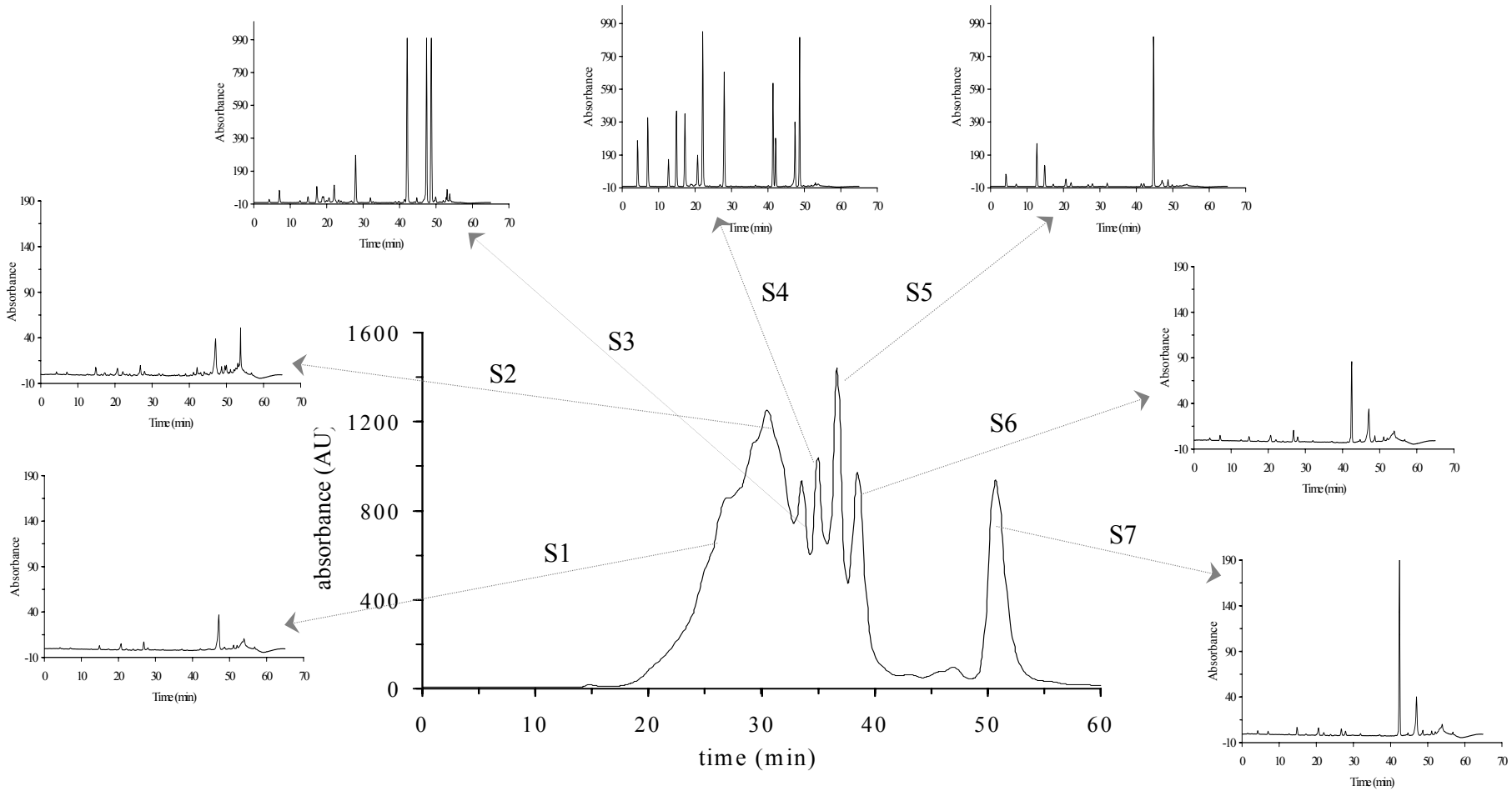


Fig. 5.7 Detection selectivity of the SEC fractions by OPA derivatization method

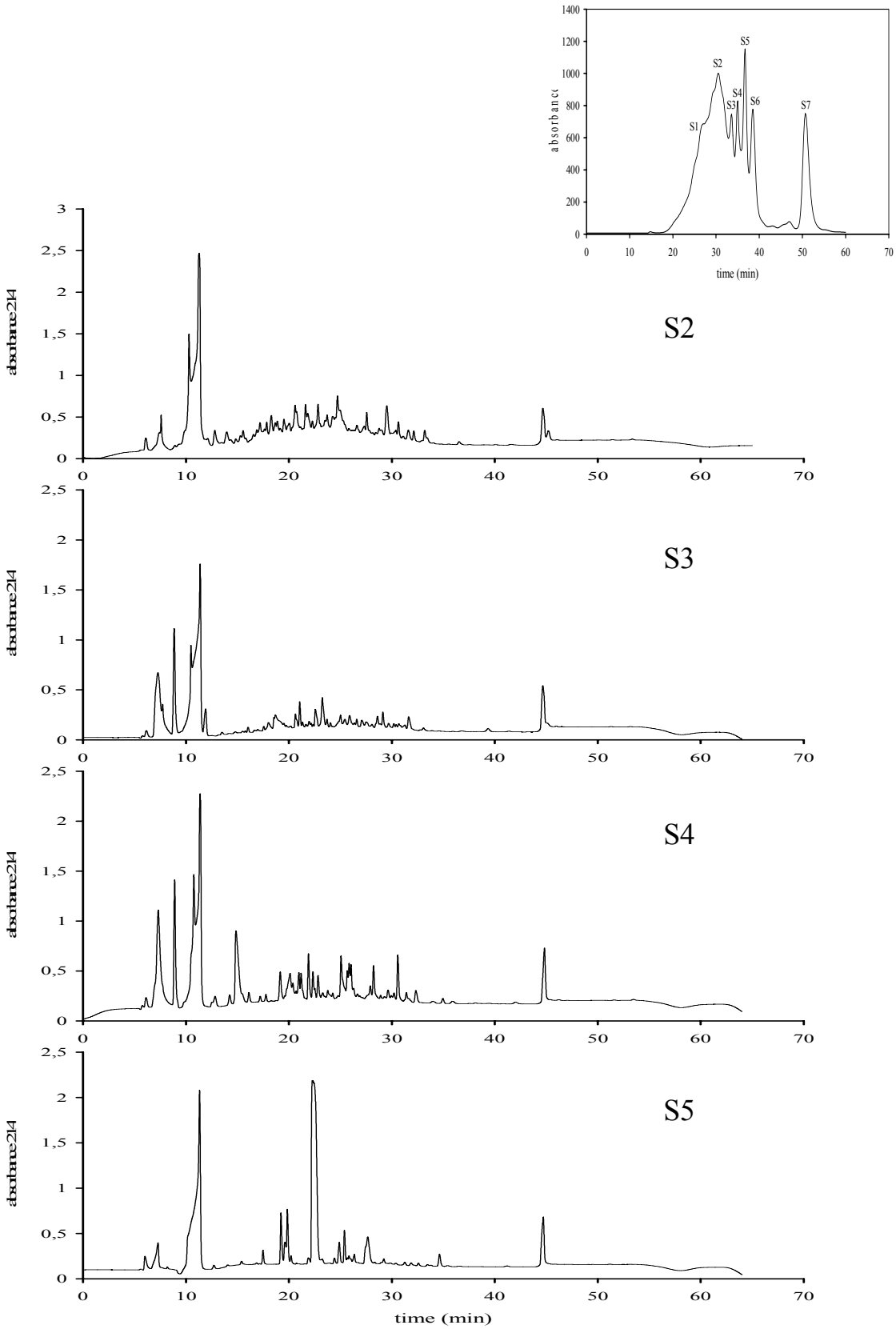


Fig. 5.8 Detection selectivity of the SEC fraction 2 to fraction 4 by non-derivatization RP-HPLC method
(the inset shows the size exclusion chromatogram)

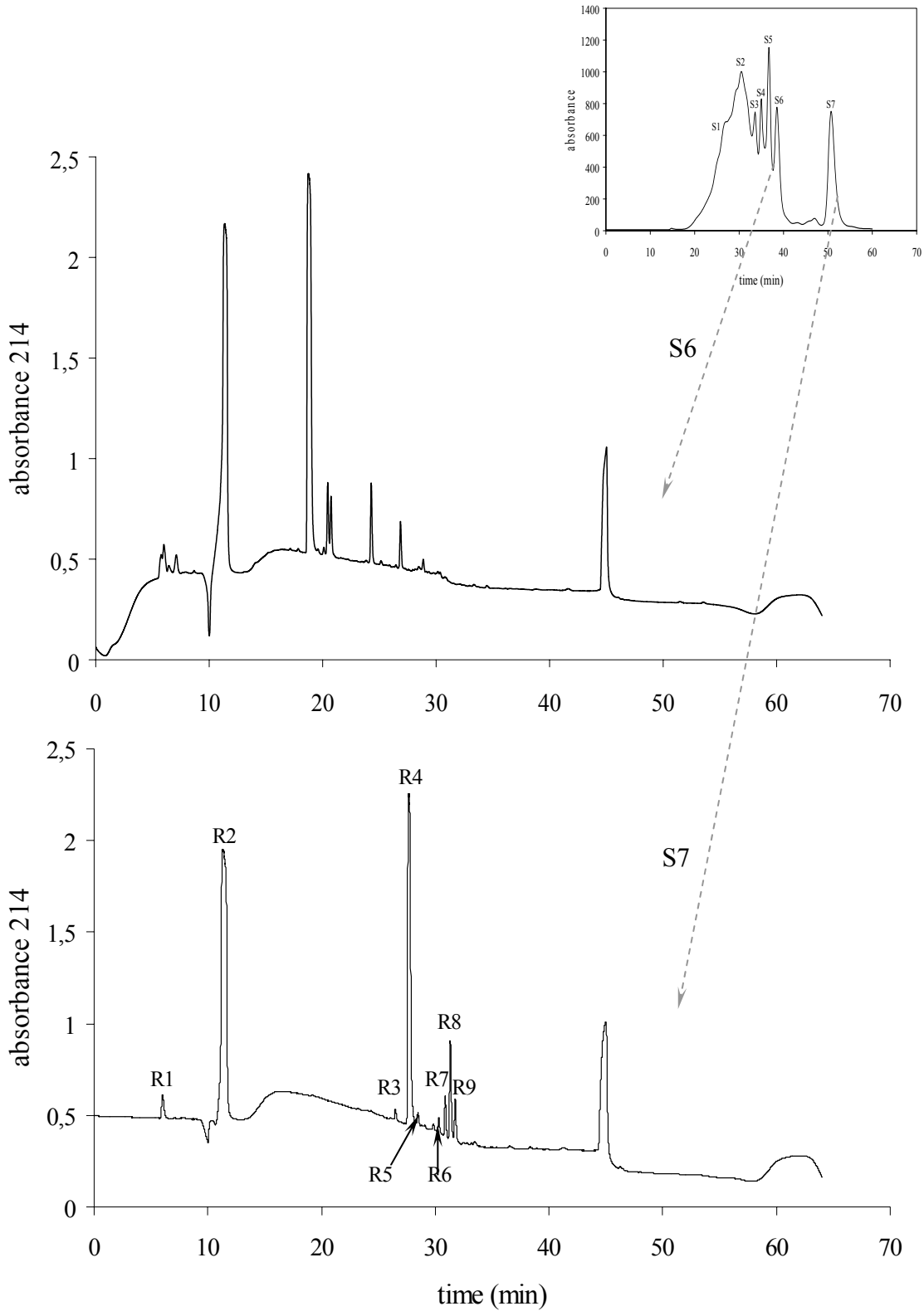


Fig. 5.8 (cont.) Detection selectivity of the SEC fraction 6 and fraction 7 by non-derivatization RP-HPLC method
(the inset shows the size exclusion chromatogram)

5.4 Conclusion

Peptide mixtures arising from enzymatic hydrolysate are usually fractionated and characterized by a combination of the chromatographic methods. Appropriate combination methods will normally yield a homogenous product. A multi-step purification scheme was developed to separate a peptide mixture in PP hydrolysates, allowing the obtained fractions to be characterized in detail. This scheme comprises the SEC on a Superdex Peptide HR10/30 column followed by OPA pre-column derivatization or non-derivatization RP-HPLC. SEC is a widely used method to separate the peptides from the protein hydrolysates by their different molecular weights. Significantly incorrect results are produced with both the small peptide and the amino acid standard mixtures since a non-ideal size exclusion effect has been observed. This effect mainly results in interactions of specific peptides/amino acids with the column matrix and electrostatic interactions with themselves, especially small peptides and amino acids, e.g. Phe, Tyr, Trp, and Angiotensin II in the standard peptides mixture.

In order to reduce non-ideal size exclusion effects it is necessary to use the higher ionic strength mobile phase, e.g. PBS, to avoid weak electrostatic interactions. On the other hand, increasing the ionic strength promotes the formation of hydrophobic interaction, while the use of less salt or the comprising of a solvent such as acetronitrile in the mobile phase can be avoided in this interaction. Since not only the co-elution of peaks 2, 3, 4, and 5, but also high salt containing fractions were obtained when eluted with PBS, these problems can be eliminated by eluting with 20 % ACN + 0.1 % TFA, which separated PP hydrolysate into seven fractions. No amino acids were contaminated in the fractions S1 and S2, while amino acids contamination was obtained in fractions S3 to S7, especially tryptophan in S7. Therefore, the selectivity separation of low molecular contaminants from peptides is difficult as a result of a similar molecular size. They are undesirable in SEC, since they are unpredictable. Thus, this method suites better for the comparison of apparent molecular weight distributions than for the precise determination of the molecular weights of protein hydrolysates.

It is unlikely that a single separation method will be able to resolve all constituents in the hydrolysate samples. Usually they require an initial separation into a small number of fractions by a chromatography method, e.g. SEC, followed by further separation of each fractions with a different chromatographic methods, e.g. RP-HPLC. The formation of fluorophore by the action of OPA with amino acids and peptides has provided a highly sensitive assay for these compounds by RP-HPLC. Although this fluorophore can react with peptides, it gives 10 to 20 times less fluorescence than those which react with amino acids.

Moreover, OPA fluorescence seem to depend on the presence or absence of lysine residue in the peptide chain and not on its position, i.e. two identical amino acid content peptides, one with a C-terminal lysine residue and one with an internal lysine residue, showed little difference in reactivity, while an absence lysine peptide reacted quite poorly with OPA (Joys and Kim, 1979). Thus, the separation signal of low molecular weight SEC fractions by the OPA derivatization method was superior to a non-derivatization method, especially S4 fraction, while a poorer detection signal was obtained from high molecular fractions (S1 and S2). Nevertheless, this method was not suitable since high salt containing fractions were obtained after lyophilization process. The utilization of ACN as a mobile phase for RP-HPLC can not only eliminated the high salt concentration, but can also improve the selectivity and decrease the analysis time. Satisfactory separation of fractions S6 and S7 were obtained. The S7 fraction contains hydrophobic peptides.

CHAPTER 6 PEPTIDE CHARACTERIZATION

6.1 Introduction

RP-HPLC has been widely used for the analysis of peptide mixtures produced by enzymatic digestion of proteins (Chang et al., 1997). However, peptide fragments have to be isolated and characterized by different approaches, such as molecular weight, amino acid composition, and sequencing analysis, that cannot be accomplished by RP-HPLC. The combination of HPLC and mass spectrometry (LC/MS) is one of the most powerful techniques for the characterization of peptides and proteins, since mass spectrometry allows not only the precise determination of the molecular weights but also the determination of the sequences (Dreisewerd, 2003; Burlingame et al., 1996). Fast atom bombardment (FAB), electrospray ionization (ESI), and matrix-assisted laser desorption/ionization (MALDI) are the most often used ionization methods to study the peptides and proteins through mass spectrometry. ESI and MALDI ion sources have become the most important methods and provide a higher sensitivity and a broader range than the FAB technique.

The analysis and characterization of the peptide fragment mixture can be performed most efficiently by HPLC directly linked to ESI. This technique has proven to be successful for the separation and characterization of enzymatic fragments in peptide mapping of proteins (Banks, 1996; Mock et al., 1993). ESI usually produces multiply charged ions $(M + nH)^{n+}$ where several charge states are present for some peptides due to the result of the electrochemical process and the charge accumulation in the droplets. Therefore, ESI spectra are often more complex than those generated by MALDI (Lin et al., 2003). Furthermore, the sample is in the solution, thus the effect of solution equilibria and solution chemistry in the produced spectrum are highly important. The capacity of the droplets to be charged by the potential source depends on the composition of the substance and the pH of the solution. The charged sites of the peptide or protein form the functional groups of the amino acids (Lewis et al., 2000). Among 20 common amino acids, only five have charged polar side chains. Arginine (Arg), histidine (His), and lysine (Lys) have positively charged side chains, while aspartic acid's and glutamic acid's side chains are negatively charged. The charged polar side chains enable the potential source to charge the droplets. Typically, the protein will carry one charge per 1000 Da, or less if there are very few basic amino acids. Small molecules with less than 1000 Da will mainly produce monocharged ions. Moreover, ESI can be used for molecules without any ionizable site through the formation of sodium, potassium, ammonium, chlorine, or acetate etc. (Hoffmann and Stroobant, 2002).

MALDI is a relatively gentle ionization technique like ESI which uses energy from lasers rather than electrical potential to ionize biomolecules. It uses small UV absorbing molecules to co-crystallize with peptides or proteins on a sample plate (Hillenkamp et al., 1991). Ionization occurs when these matrix molecules absorb the energy provided by a laser (usually 337 nm). The released energy causes a rapid matrix thermal expansion and analyte into the gas phase. The transfer of proton from the analyte to the matrix may result in charge reduction to the singly charged $[M + H]^+$ ion observed in the gas phase (Kruger et al., 2001). MALDI predominately produces $[M + H]^+$ ions. The proton is attached to the basic site of the peptide, in order to decrease basicity, whereas ESI may add one or more protons to the side chains of Arg, Lys, or His residue, or to the N-terminus amine group (Harrison, 1998). Thus, MALDI generates simple spectra for individual species and peptide mixtures. At low analyte concentrations MALDI is less sensitive than ESI regarding organic and inorganic sample impurities, such as salt in the buffer which is commonly used in the separation of the peptides (Spengler et al., 1993). Since MALDI depends on pulsed laser radiation, ions are created discontinuous. Therefore it requires mass analyzers with the capability to analyze intermittent created ions. The most common mass analyzer used with MALDI is time-of-flight (TOF).

MS can give qualitative and quantitative information on the elemental, isotopic, and molecular composition of the samples. Thus, in this study the masses of the peptides in the SE-RP separated fractions were then analyzed by MALDI and LC/ESI-MS on the positive mode to obtain the peptide mass finger print.

6.2 Materials and Methods

6.2.1 Chemicals

Acetronitrile (ACN, gradient grade), Trifluoroacetic acid (TFA, Uvasol) were purchased from Merck (Darmstadt, Germany) and α-cyano-4-hydroxycinnamic acid from Sigma-Aldrich (Steinheim, Germany).

6.2.2 Equipments

- *MALDI-TOFMS*: The Voyager DE™ Pro mass spectrometer (Applied Biosystems, Weiterstadt, Germany) was equipped with a nitrogen laser used at 337 nm. Data acquisition and analyzes were performed using Data Explorer version 4.0 software and Voyager version 5.0 software (both Applied Biosystems, Weiterstadt, Germany).

- *HPLC-ESIMS system:* The HPLC-ESIMS system consisting of the MicroPro LC pump (Eldex, Napa, CA, USA) was equipped with the Endurance autosampler (Spark, Emmen, The Netherlands) and controlled by accompanying Endurance/Midas 2.07 software (SCPA, Weyhe-Leeste, Germany). An electrospray ionization (ESI) ion trap mass spectrometer LCQ

classic (Thermo Electron, Dreieich, Germany) was used as an online mass selective detector.

6.2.3 Methods

- *Sample preparation:* The PP hydrolysates resulting from enzymatic hydrolyzation with 2 % Alcalase + 5 % Flavourzyme (w/w) was separated by SEC into seven fractions. Each fraction was further separated by non-derivatization RP-HPLC methods (as described in chapter 5). The eluate peaks were pooled separately (S_nR_m, where n = SEC fraction number and m = RP-HPLC fraction number) and concentrated by freeze drying for 50 times.

- *MALDI-TOFMS:* An experiment was performed using a matrix of α-cyano-4-hydroxy-cinnamic acid which was dissolved in a 50 % (v/v) mixture of ACN/0.1 % aqueous TFA in a concentration of 2.5 mg/ml. The equal volumes (1 μL) of matrix and the concentrated SE-RP fraction samples were mixed on a stainless steel multiple sample tray according to the dried drop technique. MALDI-TOF measurements were performed in the linear positive ion mode. The length of the flight tube was 1.2 m. A nitrogen laser at 337 nm was used. The accelerating voltage in the ion source was set to 20 kV.

- *LC/ESI-MS:* The concentrated SE-RP fraction samples were separated on the Jupiter C_{18} (300 °A, 5 μm, 150 X 1 mm) column (Phenomenex, Aschaffenburg, Germany) protected by C_{18} ODS (4 X 2 mm) precolumn (Phenomenex, Aschaffenburg, Germany). The injection volume was 20 μL. Eluent A consisted of 0.6 % (v/v) aqueous TFA, and eluent B consisted of 80 % (v/v) ACN/0.05 % (v/v) TFA. Gradient elution at the flow rate of 20 μL/min at ambient temperature was operated. The eluates of the separation step were directed to the ESI ion trap mass spectrometer (MS) and were analyzed in the full scan positive charged mode of mass range 200 to 1200 Da under optimized settings determined by flow injection analysis before: N_2 sheath gas flow, 61 arbitrary units (arb); auxiliary gas flow, 0 arb; electrospray voltage, 4.0 kV; temperature of heated capillary, 150 °C; capillary voltage, 33 V; tube lens offset 35 V; collision energy, 25 %; and activation time, 30 ms.

6.3 Results and Discussion

The SE-RP chromatograph fractions of the peptide pool analysed under positive ionization mode of MALDI resulted in unsatisfactory spectra (these spectra are not shown here). It is obvious that the samples contain a large number of compounds (perhaps peptides) in varying concentrations. Only the compounds of a higher concentration can be directly recognized, whereas those of lower concentration are mostly hidden by overlapping and by a low signal-to-noise level. This phenomenon is characterized by the suppression of molecular ion species of some compounds (peptides?) when there are others present in the mixture. The signal corresponding to these compounds may disappear completely, and thus they may not

be detected even though they can yield an easily detectable signal when being analyzed individually (Cohen and Chait, 1996; Pusch and Kostrzewa, 1996). Because TOF has a low mass resolution, this effect can especially be observed in MALDI-TOF rather than in ESI-MS. Hence, the mass resolution of MALDI-TOF was poor since the ions were formed with a broad kinetic energy distribution (Zhou et al., 1992) and a mass-independent initial velocity (Pan and Cotter, 1992). The detection mass range of MALDI lies between 500 and 350,000 Da (Russell and Edmondson, 1997). Therefore, the detection signal of the ions below 500 Da must have a very confusing interference derived from MALDI matrix ions. Low molecular mass ions can be measured if the amount of the sample is sufficient (usually ca. >10 pmole). In general, small molecules can better be analyzed by ESI which provides valid data in the range of 30 to 80,000 Da.

Peptides and proteins are sensitive to reactive oxygen species that lead to an oxidation of certain amino acid residues. Within the peptides and proteins the sulfur-containing amino acids cysteine and methionine are the most sensitive to oxidation by reactive oxygen species (Vougier et al., 2004). The oxidized peptides in the S7R3, S7R5, S7R6, S7R7, and S7R8 fractions (Fig. 6.1) were detected by MALDI analysis as a major ion peak $[M + H]^+$ at the *m/z* 850. The *m/z* shifted to 866 ($\triangle$ *m/z* = 16) corresponds precisely to the addition of one oxygen atom $[M + O]^+$ to the cysteine or methionine residue. This mass shift pattern indicates that oxidized products of both amino acids had been produced. The oxidative ion masses results were summarized in Table 6.1.

Table 6.1 Oxidation of cysteine or methionine mass ions data of the S7R3 and S7R5 to S7R8 fractions obtained from MALDI-TOF

Fraction	**$[M + H]^+$**	**$[M + O]^+$**
S7R3	850.66	866.50
S7R5	850.54	866.46
S7R6	850.19	866.20
S7R7	850.08	866.04
S7R8	850.02	866.02

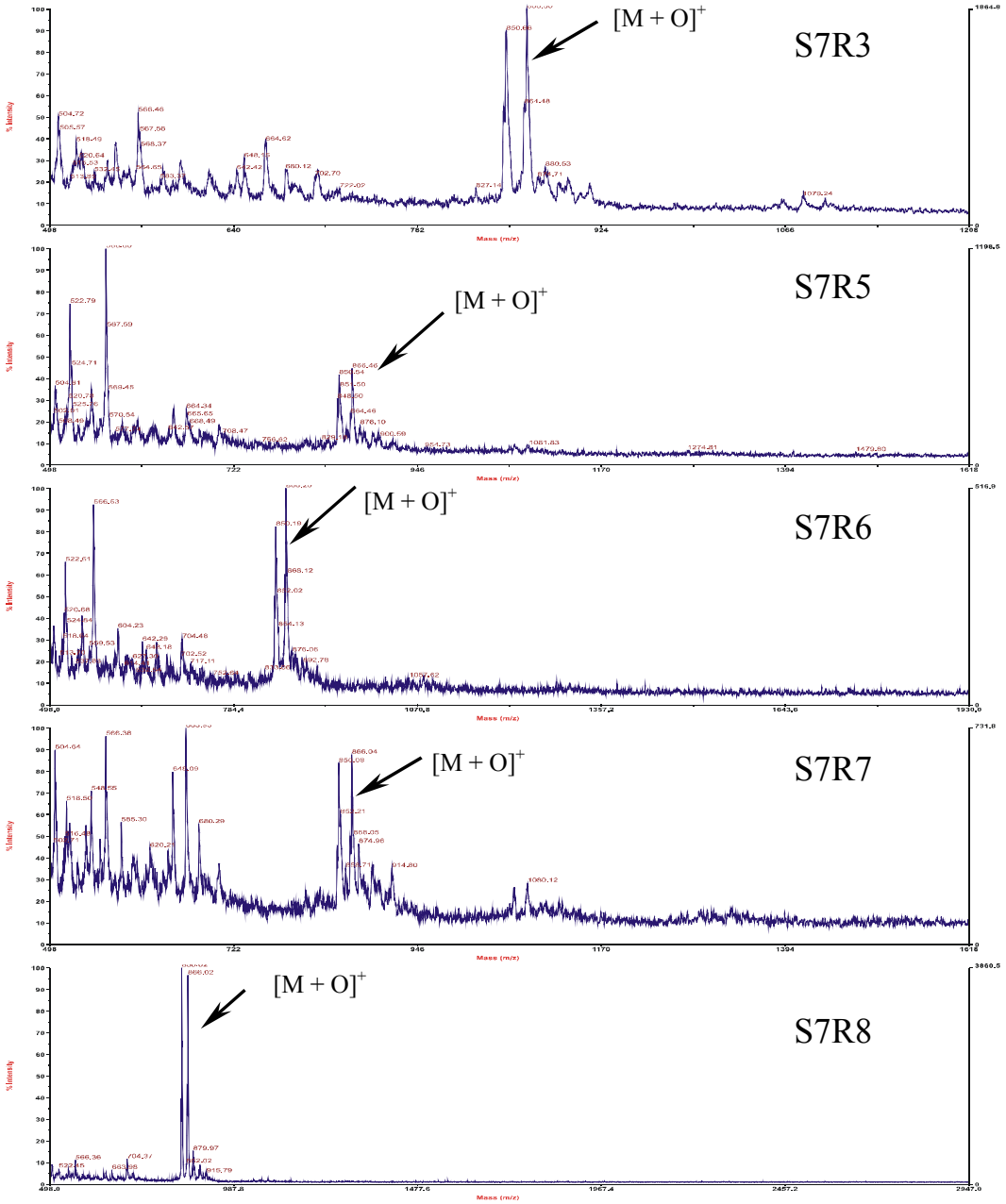


Fig. 6.1 MALDI mass spectra of the SE-RP chromatograph fractions. Peaks corresponding to the oxidation of cysteine or methionine $[M+O]^+$ ions were observed at $\triangle$ *m/z* = 16 Th (X-axis = mass (*m/z*), Y-axis = % intensity)

The electrospray ion source was easily connected to on-line liquid chromatography (LC), which allowed the analysis of complex mixtures as protein hydrolysate samples which are always dilute solutions of peptides or which contain a great number of contaminants (Burkitt et al., 2003). The S7R1 to S7R9 fractions were injected into the LC/ESI-MS and detected on the positive-ion mode as protonated molecular ions. Normally, the chromatographic conditions have to be optimized for attaining chromatograms of higher separation, but here the fractions amounts to be analyzed (S7R1 to S7R9) were very small and became crucialy insufficient making the optimization impossible. Satisfactory LC separation chromatograms were received from the S7R4 and S7R8 fractions (Fig. 6.2). Three main peaks at the retention time 19.93, 26.08, and 27.13 min, and 25.64, 29.11-31.05, and 34.20 min for S7R4 and S7R8, respectively, were obtained. These peaks were directly subjected to ESI-MS.

ESI mass spectra are usually characterized by the presence of an intense ion or a molecular ion (base peak) that represents the molecular formula of the interested compound (McLafferty et al., 1999). Normally they correspond to a statistical distribution of consecutive peaks characteristic of multiplying charged molecular ions obtained through protonation $(M+nH)^{+n}$. If manual interpretation is used to determine the protonated precursor peptide mass, and if a peptide sequence mass is known, then its monoisotopic mass can be calculated using the most abundant isotope mass of each peptide. But in the most cases the peptide sequence is unknown. Nevertheless, monoisotopic precursor peptide masses may be estimated from mass-to-charge data contained in the mass spectrum (Bakhtiar and Tse, 2000). The mass spectrum is measured as *m/z* values, the charge state of the ion can be determined from the spacing of the peaks within the isotope cluster. Assuming that the positive ion series represents different protonation states, the *m/z* of the successive peaks can be denoted P_1 and P_2, corresponding to $(m/z)_1$ and $(m/z)_2$, respectively. Thus, in order to deconvolute the spectra and to obtain the molecular weight (MW) of the peptide the charge state (z) identification of each individual signal can be achieved easily by setting up two equations and two unknowns using at least two adjacent signals in the spectrum when $P = m/z$:

$$P_1 = (MW + z_1) / z_1$$
$$P_2 = [(MW + (z_1 - 1)] / (z_1 - 1)$$

For example, the LC/ESI-MS spectrum of peak 2 at the retention time 26.16 min of the S7R4 fraction (S7R4LC2) (Fig. 6.3 B), given $P_1 = 421.0$, and $P_2 = 840.1$:

$$421.0\, z_1 = MW + z_1$$
$$840.1\, (z_1 - 1) = MW + z_1 - 1$$

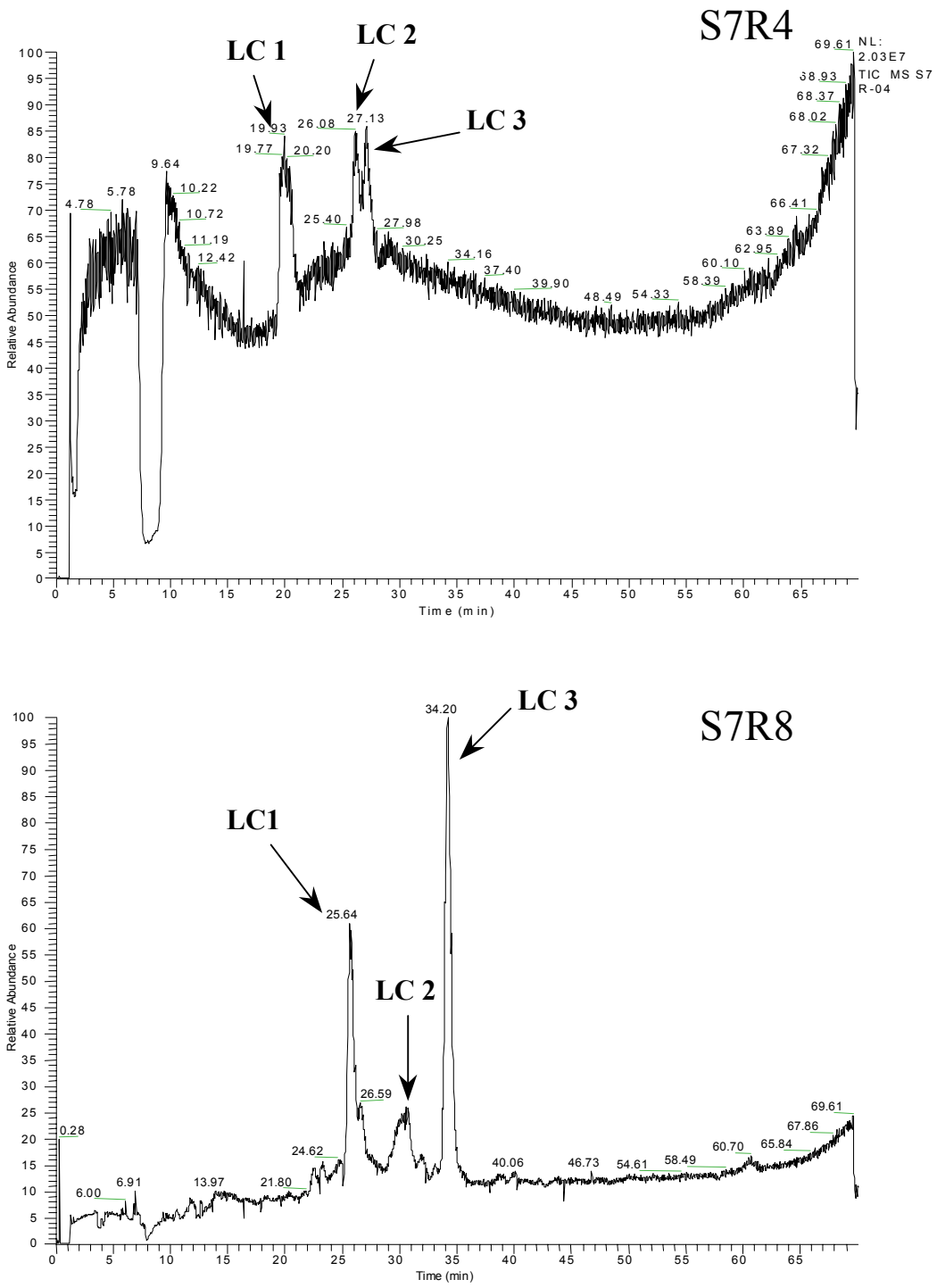


Fig. 6.2 LC/ESI-MS chromatograms of the S7R4 and S7R8 fractions (X-axis = time (min), Y-axis = Relative Abundance)

Solving for z_1 and MW yields values of 2 and 842 Da, respectively. In this spectrum something was wrong at the *m/z* 522.6 ion. It must be an impurity or contaminant peptide since the derivable z_1 value was not an integral unit (z_1 = 2.64). Thus, the mass calculation using *m/z* 840.1 and 421.0 should be skip of the *m/z* 522.6. Therefore, the abundant peak was observed at *m/z* 840.1 corresponding to the $[M + H]^+$ ion and the doubly charged $[M + 2H]^{2+}$ ion for the *m/z* 421.0 with a molecular mass of 842 Da. This doubly charged ion was also found in the spectrum of the S7R4 LC/ESI-MS of peak 3 (S7R4LC3) at the retention time 27.13 min (Fig. 6.3 C). Considering the spectrum of S7R8 LC/ESI-MS of peak 1 (S7R8LC1) at the retention time 25.64 min (Fig. 6.4 A), the single peptide with doubly charged $[M + 2H]^{2+}$ ion species at the *m/z* 840.8 was detected as well. These observations indicate that the S7R4LC2, S7R4LC3, and S7R8LC1 fractions contain peptides of the same molecular mass but of different hydrophobicity. The S7R8LC1 peptide presented higher hydrophobicity and higher signal response than others due to the physicochemical difference of the peptides, especially hydrophobicity. The peptides' hydrophobicity has been identified as an important parameter determining the ESI signal response, with higher hydrophobicity yielding higher signal and providing better peptide detection (Nielsen et al., 2004).

Invariably, smaller signals are found in a narrow range at higher mass from the base peak, and these ions of lower intensity are almost the isotope species of the molecular ion. The natural carbon is a mixture of 98.90 % ^{12}C and 1.10 % ^{13}C isotope which has an occurring isotope with one atomic mass unit (= Dalton) difference (Wehofsky et al., 2001). Hence, the other ion with lower intensity (ca. 25 % of the *m/z* 421.0) at *m/z* 422.0 ($\triangle$ *m/z* = 1) was observed in the spectrum (Fig. 6.3). This represents the molecule in which the ^{12}C atom has been replaced by the ^{13}C atom. Moreover, the distinguishment between two peptides with one mass unit difference discloses identical residues plus an extra internal amino acid pair, e.g. Asn-Ile, Asn-Leu, Asp-Asn, Glu-Gln, and Glu-Lys (Wada, 2000). These phenomena were detected from the mass spectra of the S7R4LC2 and S7R4LC3 at the *m/z* 840.1 and 840.6 ions, respectively (Fig. 6.3 B and 6.3 C). The ion masses were summarized in Tabels 6.2 and 6.3.

The S7R8LC/ESI-MS of peak 2 (S7R8LC2) and peak 3 (S7R8LC3) spectra (Fig. 6.4 B and C) showed the oxidation of the cysteine or methionine ions $[M + O]^+$ at the *m/z* 850.6-866.6 and 867.6-882.6 (S7R8LC2), and 852.7-868.6 (S7R8LC3). In addition, the S7R8LC2 spectrum also contains the corresponding ions at the *m/z* 902.6 and 925.5. This ion was shifted by 22.9 Thomson (Th), indicating the ionization by adduction of a sodium ion $[M + Na]^+$ instead of a proton. This adducted ion is quite common in ESI. Due to the positive

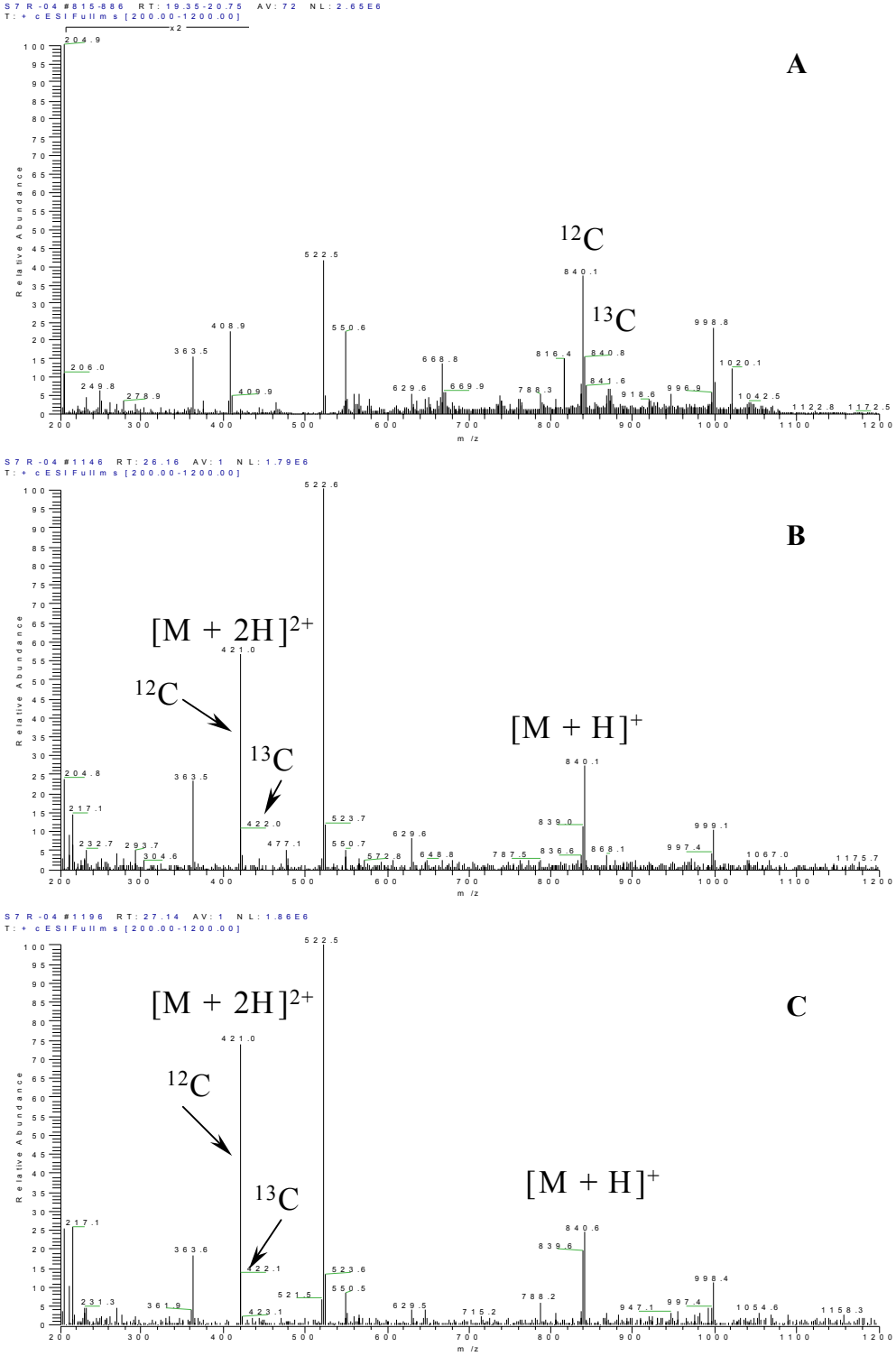


Fig. 6.3 LC/ESI-MS mass spectra of the S7R4 fraction. Peaks corresponding to the $[M+H]^+$, $[M+2H]^{2+}$, and the ^{13}C isotope ions were observed. A = S7R4LC1, B = S7R4LC2, and C = S7R4LC3 fraction (X-axis = *m/z*, Y-axis = Relative Abundance)

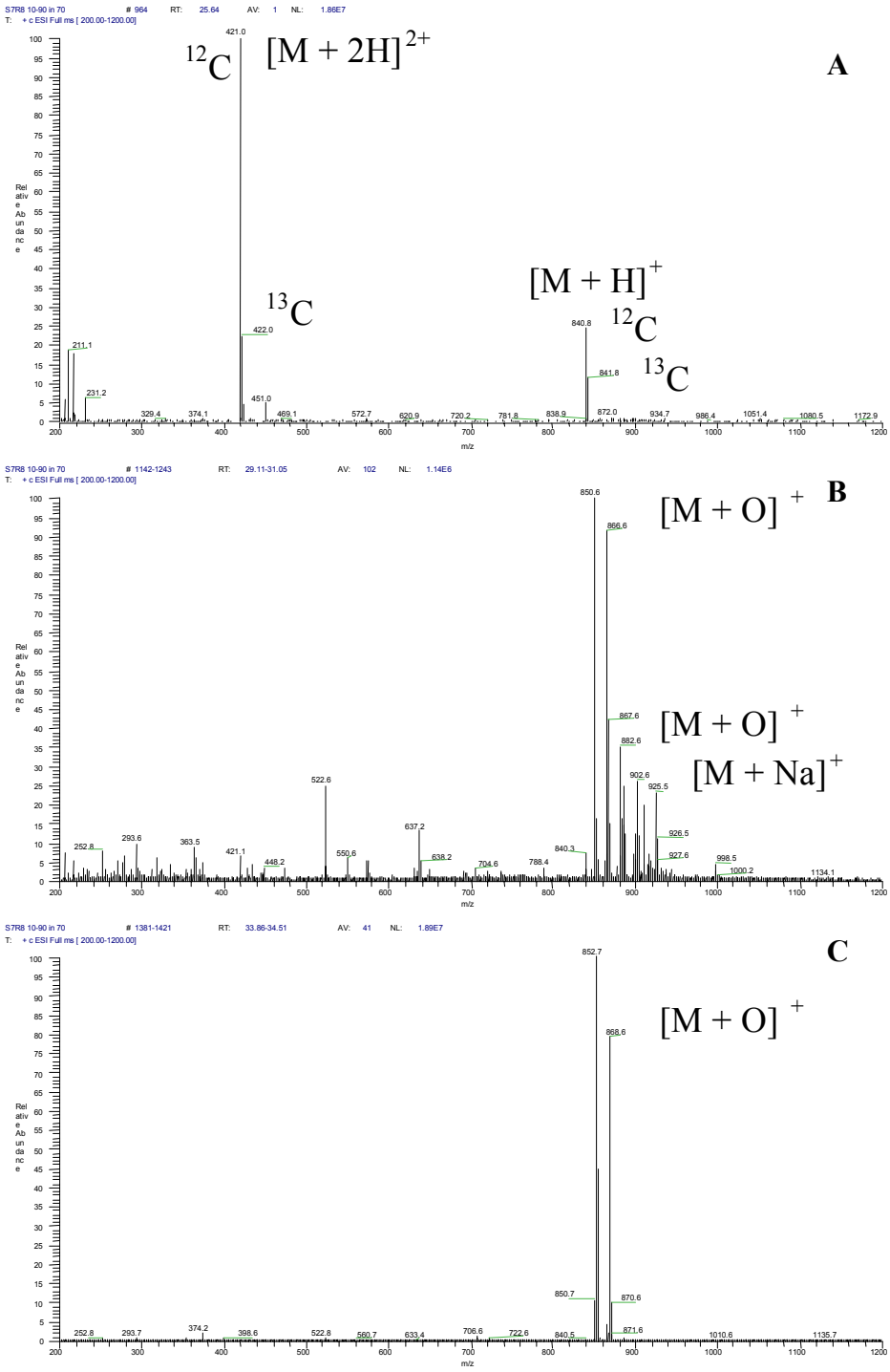


Fig. 6.4 LC/ESI-MS mass spectra of the S7R8 fraction. Peaks corresponding to the $[M+H]^+$, $[M+2H]^{2+}$, ^{13}C isotope ions, sodium adduct ions $[M+Na]^+$, and the oxidation of cysteine or methionine ions $[M+O]^+$ were observed. A = S7R8LC1, B = S7R8LC2, and C = S7R8LC3 fraction (X-axis = *m/z*, Y-axis = Relative Abundance)

detection mode for protonated molecular ions, several molecules have been ionized by the addition of the cations instead of the protons (H^+). The other adductive cations include potassium (K^+) and ammonium (NH_4^+), resulting in mass shifts 39 and 18 Th, respectively, for singly charged ions (Yang et al., 2002).

Table 6.2 Summary of the LC/ESI-MS mass ions obtained from the S7R4 fraction

Fraction	C-isotope ions		Multiply charged isotope ions	
	^{12}C	^{13}C	$[M + H]^+$	$[M + 2H]^{2+}$
S7R4LC1	840.1	840.8	-	-
S7R4LC2	421.0	422.0	840.1	421.0
S7R4LC3	420.0	422.0	840.6	421.0

Table 6.3 Summary of the LC/ESI-MS mass ions obtained from the S7R8 fraction

Fraction	C-isotope ions		Multiply charged isotope ions		Oxidated ions		Sodium adduct ions	
	^{12}C	^{13}C	$[M + H]^+$	$[M + 2H]^{2+}$	$[M + H]^+$	$[M + O]^+$	$[M + H]^+$	$[M + Na]^+$
S7R8LC1	840.8 421.0	841.8 422.0	840.8 -	421.0 -	- -	- -	- -	- -
S7R8LC2	- -	- -	- -	- -	850.6 867.6	866.6 882.6	902.6	925.5
S7R8LC3	868.6	870.6	- -	- -	852.7	868.6	- -	- -

6.4 Conclusion

MS is an important and popular method for the characterization of peptides. The two primary methods for ionization are MALDI- and ESI-MS. Different molecules have different masses, and this fact is used by the mass spectrometer to determine what molecules are present in the sample. To obtain a proper spectrum, not only a mass spectrometer with sufficient mass range and resolution, but also profound skills in peptide purification are needed. For both MALDI and ESI, the sample concentration and the complexity of the contaminants play an important role in obtaining both sensitivity and mass accuracy. The contaminants are derived from different sources such as buffer, non-volatile salts, and many

unknown original compounds. The sample dilution and contaminants problems are not easy to handle, especially when the total amount of sample is low. Therefore, it would be very difficult to interpret the results due to the overwhelming number of mixture compounds in varying concentrations that would be presented in the SE-RP fractions. An analysis of complex mixtures as PP hydrolysates is difficult because of the phenomenon of competitive ionization. Thus, unsatisfactory results were obtained from MALDI analysis since the detection signals presented the signal suppression effect and were low in respect of the signal-to-noise level, too.

The ESI-MS analysis is more sensitive than the MALDI-MS analysis. It depends on the concentration and not on the total quantity of the injected sample. Satisfactory information on the peptides contained in the S7R4 and S7R8 fractions were obtained. The LC/ESI-MS spectra of S7R4LC2, S7R4LC3, and S7R8LC1 fractions represent peptides with identical molecular mass (842 Da) but with different hydrophobicity. Furthermore, a one mass unit difference between the ions was detected. This resulted out of (i) the replacement of a ^{12}C atom by a ^{13}C atom, or (ii) two peptides having identical residues but a one single mass unit difference of their internal amino acid pairs. The oxidated cysteine or methionine ions were recognized at the *m/z* 850.6-866.6 and 867.6-882.6 for the S7R8LC2 spectrum and the *m/z* 852.7-868.6 for the S7R8LC3 spectrum. The mass shifted by 22.9 Th at the *m/z* 902.6 and 925.5 of the S7R8LC2 spectrum, indicating the sodium adducted ion $[M + Na]^+$.

CHAPTER 7 CONCLUSIONS

Interest in protein hydrolysates has increased during the past decade since it has shown that partially hydrolyzed proteins can produce hydrolysates which contain a predominance of short chain peptides. They can be utilized more efficiently and have a higher nutritive value than an equivalent of free amino acid mixtures due to the small intestine that can absorb di- and tripeptides more rapidly than free amino acids. Thus, the reaction condition of protein hydrolysis (number of enzyme, E/S, and time) should be controlled to obtain a molecular weight distribution compatible with the desired end use of the product.

The enzymatic hydrolysis of potato pulp (PP) was carried out by using four different enzymes, i.e. Alcalase (ALC), Novo Pro-D (NPD), Flavourzyme (FLA), and Corolase (COR), and eight combination of proteolytic enzyme systems in a 4 l batch reactor at 50 °C for 26 h without pH control. The Endoprotease ALC and exopeptidase FLA are most suitable for hydrolyzing the PP protein, whereas the endoprotease NPD and exopeptidase COR present a very low hydrolyzation activity for this protein type. The immediate initiation of hydrolyzation, the rapid increase of the hydrolyzation rate, and the highest degree of hydrolysis (DH), 22 %, were observed in the presence of FLA, whereas the lowest DH was obtained from NPD and COR. To improve the hydrolyzation process the combination of an endoprotease, ALC or NPD, and an exopeptidase, FLA, are more efficient than using the individual proteases. The amount of FLA influences the DH and the obtained end products. A 44 % DH was obtained from the combination of 2 % ALC + 5 % FLA (w/w). The lowest DH, 17 %, was obtained from a 2 % NPD + 5 % COR (w/w) system and a 3 % NPD + 4 % COR (w/w) system, as well. It can be used to improve the quality of PP proteins because the amino acids concentration in the enzymatic hydrolysates is significantly higher than in the native PP, especially aromatic amino acids (His, Phe, Trp, and Tyr) and the sulfur-containing amino acid Met. For many free amino acids the first four hours after adding FLA seem to be the most important period. Deamination of Asn and Gln were recognized at the long time hydrolysis. The amount of free amino acid Leu was the highest one produced: approximately 18 % of the total amount of free amino acids (Σ faa) for each enzyme combination system. From the twentieth hour of hydrolysis only a low increase in the Σ faa was observed. The highest amount of Σ faa, 306 mg/g protein, was obtained from the combination of 2 % ALC + 5 % FLA (w/w).

Enzymatic protein hydrolysate samples are often complex mixtures which contain a number of individual compounds, e.g. peptides and amino acids. The separation of such samples poses a real challenge. When the analyst is not so fortunate and no comparable

published method is available, a completely new method has to be developed. There are many approaches to method optimization. The goal of each of them is to minimize the time and to generate an optimal separation. Since amino acids are the fundamental units of peptides, the chromatographic behavior of a particular peptide will be determined by the number and properties (polarity, charge potential) of the residue side chains which it contains.

Proper particular peptide separation depends on the correct choice of column(s) and chromatographic conditions. The proper column selection will simplify the optimization of chromatographic conditions. Peak sharpness in peptide separations depends not only on the column, but also on the mobile phase composition, the chromatographic conditions, and the size and charge of the peptides themselves. Thus, the influence of various experimental parameters on the RP-HPLC separation were examined. (i) Chromatographic results from four different alkyl chain lengths (C_4, C_8, C_{12}, and C_{18}) as well as from the matrix´ different particle and pore sizes were comparable. (ii) A chromatographic condition as an effect of the hydrophobic strength of organic solvents in the mobile phase and of the temperature to the peptide retention and the selectivity have been studied.

Of the four columns evaluated, it was found that the alkyl chain length and particle and pore size of the stationary phase affected the separation efficiency (resolution) but did not affect the selectivity. The separation by the Jupiter Proteo C_{12} column demonstrated the highest resolution and reproducibility for peptide mapping of PP hydrolysates. Comparatively, the Nucleosil C_4 column exhibited the poorest resolution. Based upon the results obtained, Jupiter Proteo C_{12} column was selected for PP hydrolysate peptides separation.

Good separations can be achieved by using the mobile phase which minimizes adsorption. Gradients of iso-propanol gave poorer resolution of hydrophilic peptides than gradients of acetonitrile. On the other hand the resolution of hydrophobic peptides was improved when eluted with iso-propanol gradients. Increasing the temperature leads to a decrease in mobile phase viscosity, resulting in some decrease in band dispersion and improvement of separation efficiency or shortening run time. Moreover, it can also affect the separation selectivity.

The determination of the amino acid sequencing of a polypeptide or protein is a relatively complex analytical process, comprising two steps: (i) the hydrolyzation of the proteins to liberate the peptides and amino acids, (ii) followed by chromatographic analysis and quantification of the liberated products. For high complex mixtures as enzymatic protein hydrolysates which contain a large number of both major and minor components, no chance in selectivity will allow the separation of all compounds of interest. It may be necessary to

carry out more than one separation method. The combination of the chromatographic methods such as SEC followed by RP-HPLC are used for fractionated peptides mixtures in the hydrolysates. Appropriate combinations of these methods will generally yield the homogeneous fractions, but this is manually repetitive and time consuming.

For separating peptides from the PP hydrolysates into high molecular mass and low molecular mass fractions, size exclusion chromatography (SEC) was used. Size exclusion behavior of free small peptides, mixed small peptides and mixed amino acids standards were assessed on a Superdex Peptide HR 10/30 column, using 20 % ACN containing 0.1 % trifluoroacetic acid (TFA). A non-ideal size exclusion effect has been observed during SEC of the standards. This effect has been attributed mainly to electrostatic and/or hydrophobic interactions of amino acids and peptides with the column matrix. The elution of hydrophobic amino acids from this column were in the order of increasing hydrophobicity, i.e. Phe, Tyr, and Trp, respectively. Trp was strongly absorbed on this column, while all other amino acids eluted earlier including Leu and Ile, which have hydrophobic side chains. The aromatic amino acids Trp, Tyr and Phe are the most likely to be responsible for interaction with the size exclusion column matrix. Moreover the standard small peptide containing Phe, e.g. Phe-Gly-Gly-Phe, eluted from the column later than at the expected retention time since non-ideal size exclusion occurred.

In order to reduce the non-ideal size exclusion effect on this column various strategies have been employed, e.g. alteration of mobile phase type and increasing mobile phase strength. Poor resolution separation of the PP hydrolysates and high salt fractions were obtained when eluted with PBS. By using the elevated solvent strength of the mobile phase as TFA and ACN, it seems to be possible to frustrate this effect. Increasing TFA concentration in the mobile phase from 0.05 % to 0.15 % resulted in an increased retention time and a better peak resolution, while a poorer resolution was achieved from a high concentration of ACN (30 %). 20 µl injection volume of the PP hydrolysates eluted with 20 % ACN + 0.1 % TFA can improve the separation resolution. The PP hydrolysates were separated into seven fractions (S1 to S7). No amino acids were contaminated in fractions S1 and S2, while fractions S3 to S7 were contaminated, especially Trp in fraction S7.

SEC fractions (S1 to S7) were further separated on RP-HPLC with σ-phthalaldehyde (OPA) derivatization and non-derivatization methods. The separation signal of the low molecular weight fractions (S3 to S7) by the OPA derivatization method was superior to the non-derivatization method, especially S4 fraction, while a poorer detection signal was obtained from the high molecular fractions (S1 and S2). The superior selectivity and the

shorter retention time were realized from the non-derivatization method by using a ACN gradient. Satisfactory separation of fraction S6 and S7 were detected. The S7 fraction mainly contains small hydrophobic peptides. These separation steps have really been a time consuming and tedious task at the micro-level.

As a general rule, the separation method should be used for both sample purification and concentration. The classical method for peptides are the SEC and RP-HPLC followed by a concentration step by lyophilization of the fractions of interest. During those steps performed with a very small amount of the sample, loss of the sample can occur, e.g. the absorption of the sample on the wall of the vial during the lyophilization process. Thus, the separation and identification of the present individual peptides still seem to be difficult. It was not capable to separate individual peptides because of their low resolution. To increase the peptides concentration in the fractions of interest the preparative scale should be employed for initial peptides separation steps.

Peptide identifications were performed by MALDI-MS and ESI-MS. The purpose of the ion source is to ionize and, in some cases, vaporize the sample. The choice of the ion source depends on the sample properties and the desired degree of ionization and fragmentation. In considering the reliability of peptide identifications by MS, a variety of sources of obtaining erroneous results have to be considered. First, there is the contamination by other substances during the initial isolation, which is difficult to avoid for low level samples. Second, there is the efficient extraction of the peptides and preparation of the sample for MS while trying to minimize the presence of salts (from the high amount of 6 N NaOH for pH adjustment before enzymatic hydrolyzation process) that may adversely affect the ionization. The analysis technique chosen should be appropriate for the sample, e.g. a highly complex mixture should not be analyzed without a separation step, either prior to analysis or on-line as in LC/MS (Baldwin, 2004).

Unsatisfactory MALDI spectra were obtained since the signal suppression effects which resulted from the mixture of compounds in the SE-RP fractions still contain. Thus, only the higher concentration compounds can be detected, whereas those of lower concentration are mostly hidden by a low signal-to-noise level. Most of them have low masses, which are not suitable for analysis by MALDI due to the matrix interference. Moreover, the oxidation of cysteine or methionine for the S7R3, S7R5, S7R6, S7R7, and S7R8 fractions was achieved by increasing the major ion peak $[M + H]^+$ at the *m/z* of 850 to 866 ($\triangle$ *m/z* = 16). This mass shift pattern indicated that oxidized products of both amino acids have been produced.

The combination of HPLC and mass spectrometry (LC/MS) offers one of the most

powerful techniques for the characterization of peptide and protein molecules through the determination of molecular weight and structure. Therefore, the characterization of the S7R1 to S7R9 fractions were performed on the HPLC system interfaced with the ESI ion trap mass spectrometer. Only S7R4 and S7R8 fractions presented the satisfactory separation LC results, especially S7R8 fraction. Three main peaks at the retention time 19.93, 26.08, and 27.13 min for S7R4, and 25.64, 29.11-31.05, and 34.20 min for S7R8 were obtained.

The spectra showed the singly charged $[M + H]^+$ ions at the *m/z* 840 approximately, whereas the doubly charged $[M + 2H]^{2+}$ ions at the *m/z* 421 corresponding to the molecular mass of 842 Da. These doubly charged ions were found in the spectra of the S7R4LC2, S7R4LC3, and S7R8LC1 fractions. This indicates that they contain the same molecular mass peptides but different hydrophobicity. Some of these peptides also exhibited a one mass unit difference from each other resulting out of (i) the replacement of a ^{12}C atom by a ^{13}C atom, or (ii) two peptides having identical residues but a one single mass unit difference of their internal amino acid pairs. The oxidated cysteine or methionine ions were recognized at the *m/z* 850.6-866.6 and 867.6-882.6 for the S7R8LC2 spectrum and the *m/z* 852.7-868.6 for the S7R8LC3 spectrum. The mass shifted by 22.9 Th at the *m/z* 902.6 and 925.5 of the S7R8LC2 spectrum, indicating the sodium adducted ion $[M + Na]^+$.

Unfortunately, sequencing of the potato pulp hydrolysate peptides, especially the peptides containing in the S7R4 and S7R8 fractions, were not determined, since not only the peptide concentration was low but also the amount of sample fractions was insufficient. To solve these problems the preparative SEC and RP-HPLC scale should be used instead of an analytical scale which has been used for the initial peptides isolation in this study. Furthermore, the application used for the hydrolysate should be examined for further study, since the use of other sources of plant protein hydrolysates have been reported, e.g. use as a supplement for amino acids or peptides in an animal cell culture media.

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