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Production of Bioactive Soy Peptides

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Titre / ***Titel*****:**

Bioactive soy peptides production

Description / ***Beschreibung*****:**

Proteins can behave as antioxidants. The purpose of this work is to create peptides from soy protein with antioxidant properties. After an enzymatic digestion of the soy protein the solution will be fractionated by membranefiltration and the antioxidant properties will be analyzed.

Objectifs / ***Ziele:***

- Investigate soy protein enzymatic hydrolysis conditions
- Explore peptide fractionation by membranefiltration
- Characterize the antioxidant activity of selected peptide fractions
- Evaluate peptide characteristics

1.1 Signature ou visa / ***Unterschrift oder Visum***	**Délais** / ***Termine***
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Production of bioactive soy peptides

Objective

Synthetic antioxidants are widely used in the food industry to prevent deterioration of food products but are suspected to cause health problems. Therefore natural antioxidants such as proteins or hydrolysates of these proteins are becoming increasingly more popular. The objective of this diploma thesis was the production of peptides from soy protein isolate with antioxidative properties using hydrolytic digestion.

Results

After an enzymatic digestion with pepsin and pancreatin the peptide concentration was measured using an OPA assay, and a fractionation through a series of ultrafiltrations performed. A 3kDa membrane filtration was followed by a 1kDa membrane filtration yielding specific peptide size fractions. The solubility of the soy protein isolate in aqueous solution was found to be 38% for both digestion steps at pH levels of 1.5 (pepsin digestion) and 7.8 (pancreatin digestion). The digestion with pepsin-only or pancreatin-only resulted in a lower peptide concentration (20mM equivalent Phe-Gly) than the digestion utilizing both enzymes (30mM equivalent Phe-Gly). The comparison of two different soy protein isolates from different suppliers showed the two substrates to be different in their TGA (Thermal Gravimetric Analysis) profiles suggesting that the two manufacturers had used slightly different production methods. A 2^4 factorial design was performed to study the effects of 4 different digestion factors on total digestion peptide yield. Of the 4 investigated factors (pepsin concentration; pancreatin concentration; pepsin time, pancreatin time) the concentration of pancreatin was shown to have the most influence on peptide yield. With a higher concentration of pancreatin, a greater amount of peptides was produced. The antioxidant properties of the filtrated hydrolysates were greater when a digestion sample with a higher concentration of peptides was used. Therefore a high concentration of pancreatin used in the digestion step yielded a greater amount of antioxidant activity in the peptides produced.

Keywords: soy protein isolate, hydrolysis, peptides, ultrafiltration, antioxidative properties

Ziel

In der Lebensmittelindustrie werden häufig synthetische Antioxidantien eingesetzt, um die Verderbung der Produkte zu verhindern. Diese Zusätze sind im Verdacht gesundheitliche Probleme zu verursachen und daher wurde die Forschung von natürlichen Antioxidantien vorangetrieben.
Das Ziel dieser Diplomarbeit ist die Produktion von Peptiden aus Soja Protein mit antioxidativen Eigenschaften mittels einem hydrolytischen Verdau.

Resultate

Nach einem enzymatischen Verdau mit Pepsin und Pancreatin wurde die Peptidkonzentration mittels der OPA Methode gemessen und eine Fraktionierung mittels Ultrafiltration mit einer 3kDa und einer anschliessender 1kDa Membran wurde durchgeführt. Die Löslichkeit von Soja Protein Isolat in einer wässrigen Lösung ist mit einem Wert von 38% nicht sehr hoch, aber an den beiden pH Werten von 1.5 (Pepsin Hydrolyse) und 7.8 (Pancreatin Hydrolyse) ungefähr gleich. Der Verdau mit nur Pepsin oder nur Pancreatin führte zu einer geringeren Peptidkonzentration (20mM) als der Verdau mit beiden Enzymen (30mM). Der Vergleich von zwei verschiedenen Soja Protein Isolaten von zwei unterschiedlichen Lieferanten zeigte anhand einer TGA (Thermal Gravimetric Analysis) Analyse ein unterschiedliches Verhalten auf, welches auf unterschiedliche Produktionsarten weist. Ein 2^4 factorial design wurde erstellt, um 4 verschiedene Faktoren für die Hydrolyse zu untersuchen. Von den vier untersuchten Faktoren (Pepsin Konzentration, Pancreatin Konzentration, Inkubationszeit von Pepsin, Inkubationszeit von Pancreatin) zeigte die Konzentration von Pancreatin den grössten Einfluss. Es wurde gezeigt, dass durch eine höhere Konzentration von Pancreatin eine höhere Menge an Peptiden produziert wird. Nach der Filtration der Probe mit der höchsten Peptidkonzentration zeigten auch die antioxidativen Eigenschaften die höchste Aktivität. Daraus kann gefolgert werden dass eine höhere Konzentration an Pancreatin zu einer höheren antioxidativen Aktivität führt.

Schlüsselwörter : Soja Protein Isolat, Hydrolyse, Peptide, Ultrafiltration, Antioxidative Eigenschaften

Objectif

Les antioxidants synthétiques sont souvent utilisés dans l'industrie alimentaire pour empêcher le déterioration des produits. Mais ces ingrédients sont potentiellement nocifs pour la santé, des travaux de recherche sont effectués pour identifier des antioxidants d'origine naturelle.
Le but de ce travail de diplôme est de produire des peptides de protéine de soja avec des propriétés antioxidantes, au moyen d'une digestion enzymatique hydrolytique.

Résultats

Après une digestion enzymatique avec les enzymes pepsine et pancréatine, la concentration des peptides est déterminé par la méthode OPA. Les hydrolysats sont ensuite fractionés au moyen d'ultrafiltration avec une membrane de 3kDa et une membrane de 1kDa. La solubilité des protéines de soja dans une solution aqueuse est relativement faible, 38%, mais est similaire pour les deux pH utilisés pour l'hydrolyse enzymatique (1.5 et 7.8) . A la fin des digestions avec uniquement la pepsine ou uniquement la pancréatine, la concentration des peptides (20mM équivalent Phe-Gly) est plus faible que lorsque que la digestion effectuée avec les deux enzymes (30mM équivalent Phe-Gly). La comparaison de deux sources différentes d'isolat de protéines de soja indique un comportement différent basé sur la concentration des peptides. Parmi les quatre paramètres étudiés au moyen d'un plan factoriel d'expérience, la concentration finale de pepsine, concentration finale de pancréatine, le temps de réaction de la pepsine, le temps de réaction de la pancréatine, la concentration de pancréatine représente l'influence la plus importante. La quantité la plus élevée de peptides a été obtenue pour la concentration la plus élevée de pancréatine. Après la filtration de l'hydrolysat avec la concentration des peptides initial la plus élevée, les propriétés antioxidantes étaient aussi les plus hautes. En conclusion, ces travaux indiquent qu'une concentration élevée de pancréatine conduit à une concentration élevée des propriétés antioxidantes.

Mots-clés : isolats de protéines de soja, hydrolyse, peptides, ultrafiltration, propriétés antioxidantes

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Abbreviations

°C	Degrees Celsius
µg	microgram
APS	Ammoniumpersulfate
ATP	Adenintriphosphate
Asc. acid	Ascorbic acid
BSA	Bovine Serum Albumine
Da	Dalton
DPPH	2,2-Diphenyl-1-picryl-hydrazyl
EC number	Enzyme commission number
EtOH	Ethanol
ESI	Electrospray Ionisation
g	gram
HCl	Hydrochloric acid
ISSP	iso-electric soluble soy protein hydrolysate
kDa	Kilo dalton
M	Molar
m^2	square meter
MALDI	Matrix assisted laser desorption/ionisation mass spectrometry
MeOH	Methanol
mg	milligram
ml	milliliter
min	minute
MS	Mass Spectrometry
MWCO	Molecular weight cur-off
m/z	mass to charge
N_2	Nitrogen
NaOH	Natrium Hydroxide
NaP	Sodium phosphate buffer
nm	nanometers
OPA	o-Phthaldialdehyde
PES	polyethersulfone
pI	isoelectric point
pK	Dissociation constant
psi	pounds per square inch
RNA	Ribonucleic acid
RP-HPLC	Reversed phase - High Performance Liquid Chromatography
RT	Room Temperature
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamid gel electrophoresis
SPI	Soy Protein Isolate
TEMED	Tetramethylethylenediamine
TFA	Trifluoroacetic acid
TGA	Thermogravimetric analysis
TOF	Time of flight
UPGMA	Unweighted pair-group method using arithmetic averages
w/w	weight per weight

2 Introduction

Soy protein is an important ingredient in the food industry. Because of its high nutritional value, amino acid content, and its excellent functional properties it is often used as a meat substitute. Soybean is a very common crop and food ingredient in North America. The U.S alone supplies about 40% of the world's soybean; about half of the crop is exported, primarily to Asia.

Soy protein is the storage protein of soybean, and constitutes approximately 60% of the total protein content in the bean. Different processed soy proteins are used as food ingredients. Soy protein concentrate, soy protein isolate and soy flour are the most common, the main difference between them being the soy protein content. Soy flour contains approximately 50% soy protein, soy protein concentrate has a protein content of at least 70%, and soy protein isolate (SPI) has at least 90% protein. [1]

Various studies have shown soy protein to be beneficial to human health. In 1999, the FDA stated that the consumption of soy proteins can lower the cholesterol level and therefore reduce the risk of coronary heart disease. Other associated advantages of the use of soy products are in the prevention of obesity, diabetes, and osteoporosis. In addition, a peptide present in soy protein has recently been proven to help prevent cancer. Bioactive peptides produced through hydrolytic digestion, have become more popular as they exhibit bioactivity. One of the most common hydrolytic digestion peptides is considered to be an antihypertensive due to the inhibition of the angiotensin converting enzyme. These facts and studies have contributed to the recent popularity of soy proteins. [2]

Antioxidants are used in food to prevent deterioration due to oxidation. More recently soy protein has been used to stabilize lipids or lipid containing foods. Synthetic antioxidants are cheap and effective but are suspected to cause health problems. Therefore the research of natural antioxidants has received attention. Different proteins show differing antioxidative activity. Soy protein, milk casein and bovine serum albumin have all been shown to have varying degrees of antioxidative activity. The hydrolysis of these proteins leads to peptides with even higher antioxidative properties. These peptides are mostly composed of 5-16 amino acids and include hydrophobic (valine, leucine) amino acids at the N-terminus and aromatic amino (Tyrosine, histidine, proline) acids in the sequence. [3]

2.1 Objectives

The purpose of this work was to produce (1) peptides from soy protein isolate with antioxidant properties, through a hydrolytic enzyme digestion using pepsin and pancreatin and (2) enriched peptide fractions by membrane filtration.

3 Theoretical Background

3.1 Soybean

The soybean is a seed, originally from China, and constitutes the base of human and animal diets in many Asian countries. Soy has a high nutritional value and a low cost, is a good source of plant proteins, and has a low fat content. The soybean contains 48-50% proteins, 20% lipids, 4-10% water, minerals (iron, copper, manganese, calcium, magnesium, zinc, cobalt, potassium and phosphorous) and vitamins (thiamin and riboflavin). Minor components considered to be anti-nutritional factors include protease inhibitors, phenolic compounds, lectin, saponins, and phytate. A majority of the anti-nutritional factors can be removed by conventional methods such as heat treatment, germination and micronization (continuous process of heat treatment that is based on short-time processing by infrared radiation). Heat treatment is a very effective process for the elimination or reduction of heat-labile anti-nutritional factors (protease inhibitors, lectins, nitrogens, and vitamins). [1]

Figure 1 Green soybeans (websource: http://www.herbal-nutrition-solutions.com/image-files/soy-beans1.jpg)

Soy proteins contain two major globulins, glycinin and β-conglycinin. glycinin has a molecular weight between 309 and 390kDa and is a hexamer consisting of two trimers. Each trimer has three acidic and three basic polypeptides paired and is held together by disulfide and hydrogen bonds. The acidic (size between 37 and 40kDa) and the basic (size around 20kDa) polypeptides are alternating. At different pH and ionic strength, the conformation of

the glycinin is different. At pH 3.8 or lower the non-structured protein dominates correlating with the quaternary dissociation of the molecule. [4] β-conglycinin has a molecular weight between 140 and 170kDa and a trimetric structure. The three different subunits are α' with a molecular weight between 57 and 83kDa, α with a molecular weight between 57 and 76kDa and the subunit β with a size between 42 and 53kDa. The pH of the solution has an influence on the charge of the globulins. Below the pI of the soy protein (4.6), the overall protein charge is positive and above the pI the charge is negative. [5]

The composition and the conformation of proteins are responsible for their functionality. Differences in composition that may alter functionality include the ratio of protein fractions, the variations in subunit concentrations within fractions, or differences in amino acid profiles. The functionality of soy protein is partly dependent on the glycinin to β-conglycinin ratio, which can vary between genotypes. As soybeans mature, the concentration of glycinin increases at a higher rate than that of β-conglycinin. [6]

Thermal behaviour of proteins is also important and can be analysed by different methods. For example, thermogravimetric analysis (TGA) is a simple analytical method to measure the loss of weight as a function of temperature. The weight change profile as a function of temperature can be used to determine the type of degradation. The derivative of this curve is used to determine the point where the loss of weight is the most apparent. The precision of this method relies on the three following factors: temperature, temperature change and weight measurement. The measurement of the weight is performed by the comparison between the reference and the sample. After thermal degradation is completed, an ash content remains, this is a useful parameter for understanding product quality. The ash content directly relates to the quality of the end product, with a low ash content constituting a higher quality product. [7]

3.2 Proteolytic Modification

Proteolytic modification of food proteins is an ancient technology. It essentially involves the improvement of the taste and the storage stability of protein resources. To accomplish proteolytic modifications of food proteins, enzymes are generally used. These enzymes may be secreted by microorganisms during a fermentation, be already present in the raw material, or as it is more common, added separately. What occurs during a controlled proteolytic modification is essentially the same as during the enzymatic hydrolysis of ingested protein. Already in the year 1940 the patients who couldn't take undigested protein, were given

protein hydrolysates for the maintenance of their nutritional status. A disadvantage is the well-known bitter taste observed in many of the protein hydrolysates. The bitter peptides characteristically contain neutral amino acids with large alkyl or aromatic side chains. However other tastes can be found in protein hydrolysates. For example, peptide esters (including aspartame) are sweet. The taste contribution from protein hydrolysates in food is often more complicated than just a question of the presence or absence of bitterness. [8]

Protein hydrolysates generally contain the same amino acid composition as the raw material. However, there is for example in the ISSPH (iso-electric soluble soy protein hydrolysate), a slightly reduced amount of hydrophobic and sulphur-containing amino acids, and a slightly increased amount of lysine. In these hydrolysates, some allergenic peptides could be present, but all in all, protein hydrolysates should be much less allergenic than the corresponding intact proteins. Soybeans contain a number of anti-nutritional factors, the most important being trypsin inhibitors. The presence of trypsin inhibitors in soy beans lowers digestibility. Phytic acid is another anti-nutritional component of concern, as it reduces the bio-availability of calcium and zinc. In ISSPH, the content of phytic acid is very low and thus not of any concern. [8]

To characterise the amount of peptides produced during an enzymatic proteolytic modification different methods can be used. One method is the OPA (o-phthaldialdehyde) assay. The released amino groups during hydrolysis react with the OPA and β-mercaptoethanol and form a compound that absorbs at 340nm, allowing the produced peptides to be monitored by spectrometry. [9] [10]

3.3 Antioxidative Peptides

Peptides consist of amino acids. They can be produced enzymatically or synthetically. An enzymatic method to produce peptides is to cleave a protein by a protease such as pepsin (predominant digestive enzyme in the gastric juice of vertebrates). Peptides can be biologically active in a number of different ways. An example is the β-amyloid (39-43 peptide residues) that plays a role in the Alzheimer's disease. It initiates the death of neuron during the prolonged period of the Alzheimer's disease. Other peptides have known antimicrobial properties. The peptides of interest in this thesis are of an antioxidative nature. [11]

Antioxidants are widely used as food additives to delay food deterioration. A molecule that is capable of slowing down or preventing the oxidation of other molecules is known as an antioxidant. Oxidation transfers electrons from a substance to an oxidizing agent and can produce free radicals, which start detrimental chain reactions that can damage cells. Effective antioxidants act by stopping the chain reactions by removing radical intermediates, and inhibit other oxidation reactions by being oxidized themselves. Antioxidants are often reducing agents such as thiols or polyphenols. Although oxidation reactions are fundamental for life, they can also be damaging. Plants and animals maintain therefore their complex systems with multiple types of antioxidants, such as glutathione, vitamin C, vitamin E as well as with enzymes such as catalase, superoxide dismutase and various peroxidases.

Ascorbic acid, also known as vitamin C, is an antioxidant found in both animals and plants. Humans can not synthesize this compound, so we take in this vitamin through food. Ascorbic acid is a reducing agent which neutralizes reactive oxygen species such as hydrogen peroxide. In cells, it is maintained in its reduced form by reacting with gluthatione. In addition to its direct antioxidant effects, ascorbic acid is also a substrate of the antioxidant enzyme ascorbate peroxidase. This aspect of the molecule is particularly important for stress resistance within plants.

Oxygen consumption can increase by a factor of more than 10 during exercise. This leads to an increase in the production of oxidants and results in damage that contributes to muscular exhaustion during and after exercise. The inflammatory response that occurs after strenuous exercise is also associated with oxidative stress. During this process, free radicals are produced by neutrophils to remove damaged tissue. As a result, excessive antioxidant levels have the potential to inhibit recovery and adaptation mechanisms. Some athletes take antioxidants to increase their performance. The intake of antioxidants seems to have a good effect before strenuous exercise and may reduce the amount of muscle damage. [12]

There are different methods to measure antioxidative properties. The majority of techniques are based either on a single electron transfer reaction or are involved in a hydrogen atom transfer reaction. Assays based on the electron transfer involve one redox reaction with the oxidant, while the assays based on the hydrogen atom transfer mostly monitor competitive reaction kinetics. Both methods are intended to measure the radical (or oxidant) scavenging capacity. As the antioxidative activity is measured by an individual assay, it reflects only the chemical reactivity under the specific conditions applied in that assay. Therefore it is

inappropriate to generalize the data as an indication of total antioxidant activity. The DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay is based on the electron transfer method. It is a stable and commercially available organic nitrogen radical and has an absorption maximum at 515nm. During the reduction reaction, the colour of the solution decreases and can be monitored spectrometrically. Because a unique standard procedure for antioxidant analysis does not exist, it is often difficult to compare results between laboratories. [13]

3.4 Enzymes

Enzymes can be defined as biocatalysts that accelerate chemical reactions. In some cases they can increase reaction speeds by a factor of 10^{12}. Nearly all enzymes are proteins. Another category of enzymes are ribozymes that consist of active nucleic acids that are necessary for example in the splicing process of RNA. An enzyme classification system exists which takes into account the specific substrate and the type of reaction. Each enzyme gets an EC number with 4 digits. There are 6 main classes in which enzymes with the same catalytic reaction specificity are merged. Oxidoreductases catalyse oxidation/reduction reactions (transfer of H or O atoms or electrons from one substance to another). Transferases transfer functional groups from one molecule to another. The formation of two products from a substrate by hydrolysis is achieved by hydrolases (example: a protease is cleaving a peptide bond). Lyases are working by non-hydrolytic addition or removal of groups from substrates. C-C, C-N, C-O or C-S bonds may be cleaved. Intramolecular rearrangements are performed with isomerases. The last group of enzymes is the ligases which carry out the synthesis of new C-O, C-S, C-N or C-C bonds with simultaneous breakdown of ATP. Because an enzyme binds to the substrate very specifically on the active site, the reaction catalyzed by the enzyme is very specific. [14]

Pepsin (EC-Number 3.4.23.1) is the predominant digestive protease in the gastric juice of vertebrates and belongs to the peptidase family in the class hydrolases. It has a molecular weight of 35kDa and cleaves only peptide bonds. It does not hydrolyze non-peptide amide or ester linkages. Pepsin cleaves hydrophobic, preferably aromatic residues. Preferably it cleaves at the carboxyl side of phenylalanine and leucine. Pepsin will not hydrolyze at valine, alanine, or glycine linkages. As an application, pepsin is commonly used to cleave antibodies. It cleaves the heavy chains near the hinge region and three fragments of the antibody will be obtained. Optimal digestion conditions are around a pH of 1 at 37°C, with deactivation of the enzyme occurring at a pH higher than 6. [15]

Pancreatin is a blend of different enzymes. It contains amylase, lipase, ribonuclease and protease. Trypsin, α-chymotrypsin, elastase I and II and carboxypeptidase A and B are the major proteases in the mix. Trypsin (EC-Number 3.4.21.4) is an endolytic serine protease and acts mostly at the carboxyl side of lysine and arginine. α-Chymotrypsin (EC-Number 3.4.21.1) is another endolytic enzyme and cleaves at the carboxyl sides of tyrosine, tryptophan, leucine and phenylalanine. Pancreatic elastase (EC-Number 3.4.21. 36) has an endolytic function and cleaves preferably at the carboxyl side of small, hydrophobic amino acids such as alanine. Carboxypeptidase (EC-Number 3.4.17.1) acts exolytic on peptides and releases the last amino acid of the chain. If a proteolytic action of the pancreatin is desired, the optimal digestion conditions are around pH 7.5 and 40°C. [16] [17]

3.5 Membrane filtration

Membrane separation of biomolecules is becoming increasingly popular as the technique allows for processing at moderate temperatures, a high recovery of the product, and no need of additional reagents. The fractionation of active biomolecules is not a simple process but can be very useful in both industrial and research settings. The choice of membrane material is often challenging as charge and the functional properties of biomolecules require consideration. [18]

The pH of the solution changes the charge of proteins, and therefore has a significant impact on protein transport during a filtration. If the pH of a protein is higher than the pI; the charge of the protein becomes negative while the charge becomes positive if the pH is lower than the pI. For this reason a negatively charged membrane is used to filtrate proteins above their pI. [19]

Membranes can be made of many different materials which will affect their behaviour. The most commonly used materials are polyethersulfone (PES) and regenerated cellulose. PES membranes allow a fast concentration or desalting of higher concentrated samples like serum or plasma, and provide an extremely fast separation because of its open microstructure. Membranes based on regenerated cellulose have the advantage that the adsorption of protein should be reduced, but a more diluted sample is required to have a good separation. The characteristics of a membrane material can be optimised to achieve particular properties. A different microstructure (open or tight) has for example a different impact of the transport of macromolecules such as proteins. A hydrophilic, negatively charged surface on a membrane will provide increased resistance to proteins, peptides or other biomolecules (with a positive

charge). Peptides may contain both hydrophobic and/or hydrophilic amino acids which will interact with the hydrophilic or hydrophobic membrane materials. Therefore a high absorption of biomolecules is often seen on membrane surfaces. The use of a membrane with a high recovery, a tight microstructure and a low possibility of adsorption is advantageous. [20]

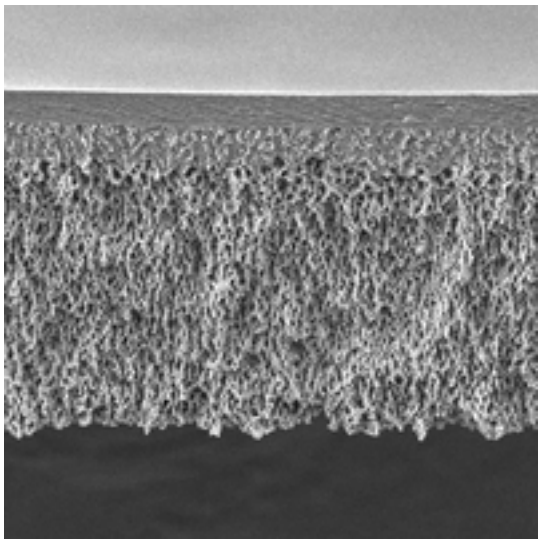

Figure 2 Ultracel regenerated cellulose ultrafiltration membrane (websource: www.millipore.com)

The ability to retain molecules is given by the MWCO of the membrane. Depending on the manufacturing process many different MWCOs are available; starting from 0.5 to 500kDa. Because there exists a size distribution of pores, retention is not absolute. Therefore the MWCO of the membrane should be around 10% higher than the biggest macromolecule in the solution being retained. [20]

3.6 SDS-PAGE

The SDS-PAGE is a method that is widely used in biochemistry, molecular biology and genetics to separate molecules according to their molecular weight. The protein solution is mixed before the separation with SDS, an anionic detergent which denatures secondary and non-disulfide-linked tertiary structures, and gives a negative charge to each protein in proportion to its mass. Without the addition of SDS, different proteins with similar molecular weights would migrate differently because of their differences in folding. SDS solves that problem as it linearizes the proteins. Because of the uniform mass to charge ratio for most proteins, the distance of migration through the gel can be assumed to be directly related to the polypeptide molecular weight. The conventional SDS-PAGE protocol, established by Laemmli, does not give a good resolution for polypeptides smaller than 20kDa. If the polypeptide range of interest is below 10kDa, another method needs to be applied. For

example, the use of tricine in the lower molecule size range is useful, as tricine has a lower pK than glycine (originally used by Laemmli) and stacks smaller molecules. [21] [22]

3.7 Mass Spectrometry

Mass spectrometry is widely used in scientific and analytical applications because of its very high sensitivity, resolution, and accuracy to determine the molecular weight of a molecule. In the case of a large molecule, such as an intact protein or strands of nucleic acids, the molecular weight can be measured to an accuracy of 0.01%. This is usually sufficient to detect minor mass changes in a molecule. In proteomics and protein chemistry, MS is used for accurate molecular weight measurement, reaction monitoring, amino acid sequencing, or protein structure determination. The analysis of proteins or peptides is usually performed using Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI MS) or Electron Spray Ionization Mass Spectrometry (ESI MS). The sample for MALDI MS is prepared as a solid crystalline deposit from which the ions are generated by laser irradiation. In ESI MS, the sample is solubilized in an aqueous/organic solvent mixture and ionized in an electrostatic spray interface.

The five basic parts of any mass spectrometer are: a vacuum system; a sample introduction device; an ionization source; a mass analyzer; and an ion detector. The mass spectrometer determines the molecular weight of chemical compounds by generating, separating, and detecting molecular ions according to their mass-to-charge ratio. Gas-phase ions are produced from a solid (in MALDI) or liquid (in ESI) sample in the ionization source by inducing the loss or the gain of a charge by neutral molecules. If the ions are formed in the gas phase, they can be electrostatically directed into a mass analyzer, separated according to their m/z ratio, and finally detected. The result is a mass spectrum can then provide molecular weight, or even structural, information.

Matrix assisted laser desorption/ionization was developed in the mid-1980s and provides an ideal ionization method for mass spectrometry of biomolecules. It's extensively used for protein identification by peptide mass mapping in proteomics. In the analysis, the analyte is first co-crystallized with a large molar excess of a matrix compound, usually a UV-absorbing weak organic acid, to generate a solid sample. This "solid solution" is then irradiated by a pulsed UV laser, leading to sublimation of the matrix that in the process carries the analyte with it into the gas phase. The matrix therefore plays a key role by strongly absorbing the laser energy to softly lift the analyte species into the gas phase without destroying them. To

ionize the analyte molecules, the matrix also serves as a proton donor and acceptor in the plasma (ionized gas). Intact proteins (MW > 8000Da) may generate singly protonated $[M+H]^+$ and multiply protonated $[M+nH]^{n+}$ ion species, whereas peptides in the MW range of 600 – 5000Da predominantly generate singly protonated ion species $[M+H]^+$. Following ionization, the gas phase ions are guided from the ion source into the mass analyzer that separates them by their m/z ratio. The performance of the ion optics and the mass analyzer is critical because these components determine the accuracy, resolution and range of the instrument.

In time-of-flight mass analysis (often used in MALDI analysis), ion m/z is determined by accurate measurements of ion drift time in a high vacuum. Ions travel from the ion source to the detector with a given amount of kinetic energy. Because all the different ion species have the same kinetic energy, yet a different mass, the ions reach the detector at different times due to the different velocities. Because of their higher velocity low molecular weight molecules reach the detector first. In the Time of Flight (TOF) mass analyzer, the m/z is determined as a function of the time of arrival of the ion.

The real challenge for the analysis of complex peptide or protein mixtures by MALDI is the sample preparation step prior to mass spectrometric analysis. A range of sample preparation methods and strategies have been developed to obtain the best possible spectra from peptide mixtures, intact proteins, serum samples, phosphopeptides and many others. The dried droplet method is commonly used for simple peptide or protein samples. In this method, mixing of an equal volume of analyte and matrix solution is performed on the MALDI target and the mixture is then allowed to dry in ambient air before the sample is inserted into the mass spectrometer for analysis. A common matrix is for example α-cyano-4-hydroxycinnamic acid. Another method is the thin layer method that decouples matrix deposition from sample deposition. A matrix solution prepared by using a low-viscosity, volatile solvent is deposited on the MALDI plate. Fast evaporation of this solvent results in a thin, homogeneous layer of matrix crystals. A small volume of acidified sample solution is placed on top of the thin matrix layer and allowed to dry. The sample is then quickly rinsed by adding a droplet of 0.1% TFA to the sample deposit and then incubated for a few seconds followed by removal of the solvent. This method is robust and is very well suited for peptide mass mapping applications in proteomics. [23]

4 Material and Methods

4.1 Material

Standard laboratory materials such (pipettes, beakers, Erlenmeyer flasks, etc.) were used for the realisation of the project and are not listed below.

4.1.1 Chemicals and Reagents

All chemicals, including product numbers and supplier information, used during the course of this work are listed in Table 1.

Table 1 Summary of chemicals

Chemical	Product number	Company
Acetic Acid glacial	ACS003-40	EMD Serono, Mississauga, ON, Canada
30% Acrylamid/Bis solution	161-0158	Bio Rad Laboratories, Mississauga, ON, Canada
Ammonium persulfate	161-0700	Bio Rad Laboratories, Mississauga, ON, Canada
L-Ascorbic acid 99%	A92902	Sigma-Aldrich, Oakville, ON, Canada
Asparagine	A0884	Sigma-Aldrich, Oakville, ON, Canada
Bovine Serum albumin standard (2mg/ml)	500-0206	Bio Rad Laboratories, Mississauga, ON, Canada
Bromphenol blue sodium salt	B5525	Sigma-Aldrich, Oakville, ON, Canada
Coomassie brilliant blue (G)	B0770	Sigma-Aldrich, Oakville, ON, Canada
2,2-Diphenyl-1-picryl-hydrazyl	D9132	Sigma-Aldrich, Oakville, ON, Canada
Ethanol 98%	UW	University of Waterloo, Waterloo, ON, Canada
Glycerol	ACS372-76	BDH inc.; Toronto, ON, Canada
Glycine	G7126	Sigma-Aldrich, Oakville, ON, Canada
Hydrochloric Acid	A144P212	Fisher scientific; Nepean; ON, Canada
2-Mercaptoethanol	M7154	Sigma-Aldrich, Oakville, ON, Canada
Methanol	UW	University of Waterloo, Waterloo, ON, Canada
MES solution (1M)	M1317	Sigma-Aldrich, Oakville, ON, Canada

Continuation of Table 1

Chemical	Product number	Company
Molecular weight standard, broad range	161-0317	Bio Rad Laboratories, Mississauga, ON, Canada
L-(+)-α-Phenylglycine	151834	MP biomedicals, Solon, OH, USA
o-Phthaldialdehyde 99% HPLC grade	P0657	Sigma-Aldrich, Oakville, ON, Canada
Protein Assay dye	500-0006	Bio Rad Laboratories, Mississauga, ON, Canada
Serine	S4375	Sigma-Aldrich, Oakville, ON, Canada
Sodium borate * $10H_2O$	S9640	Sigma-Aldrich, Oakville, ON, Canada
Sodium dodecyl sulphate	L4509	Sigma-Aldrich, Oakville, ON, Canada
Sodium chloride	ACS783	BDH inc.; Toronto, ON, Canada
Sodium hydroxide pellets	SX0600-3	EMD Serono, Mississauga, ON, Canada
Sodium phosphate dibasic	55136	Sigma-Aldrich, Oakville, ON, Canada
Soy protein isolate PRO FAM® 974	066974	ADM; Decatur, IL, USA
Soy protein isolate	FXP219PIP	The Solae company; St.Louis, MO, USA
Tetramethylethylenediamine	161-0800	Bio Rad Laboratories, Mississauga, ON, Canada
Tricine	T0377	Sigma-Aldrich, Oakville, ON, Canada
Tris base	X188-7	Baker Chemical Co.; Phillisburg, N.J.; USA
Urea	UX0065-1	EM Science (Affiliate of Merck), Darmstadt, Germany

4.1.2 Equipment

All equipment, including model and supplier information, used during the course of this work are listed in Table 2.

Table 2 Equipment summary

Equipment	Company
Balance type 1801 (110g-0.1mg)	Sartorius; Mississauga; ON; Canada
Table centrifuge 5415	Eppendorf; Mississauga, ON, Canada
Electrophoresis System Mini-PROTEAN® 3 Cell	Bio Rad Laboratories; Mississauga; ON, Canada
Freeze dryer Freezezone 4.5	Labconco; Kansas City, MI; USA
MALDI-TOF Reflex III	Bruker Daltonics Inc.; Billerica, MA; USA
Microplatereader Multiskan Ascent	Labsystems represented by Fisher Scientific; Nepean, ON, Canada
Oven at 100°C	Hotpack; Waterloo, ON, Canada
pH Meter/controller	Chemcadet
Shaker Model G2	New Brunswick scientific, Edison, N.J., USA
Stirrer Isotemp	Fisher Scientific; Nepean, ON, Canada
Spectrometer Cary 1 Bio	Varian Canada inc.; Mississauga, ON; Canada
TGA system SDT 2960 Simultaneous DTA-TGA	TA instrument; Grimsby, ON; Canada
Power Supply HEATH	Zenith; Bristol, VA, USA
Waterbath Model G76	New Brunswick scientific, Edison, N.J., USA

4.1.3 Membranes

The membranes utilized during the course of this work are listed in Table 3. The product number, the pore size and the supplier information are included in the same table.

Table 3 Summary of membranes

Membrane	Pore size (MWCO)	Company
YM3-PLBC06210 (Regenerated Cellulose)	3kDa	Millipore, Billerica, USA
YM1-13332 (Regenerated Cellulose)	1kDa	Millipore, Billerica, USA

4.1.4 Enzymes

The enzymes utilized during the course of this work, including product numbers and supplier information, are listed in Table 4.

Table 4 Summary of enzymes

Enzyme	Product number	Company
Pepsin from porcine stomach Mucosa	P7012	Sigma-Aldrich, Oakville, ON, Canada
Pancreatin from Porcine Pancreas	P1625	Sigma-Aldrich, Oakville, ON, Canada

4.1.5 Software

The different software packages used for the execution of this project are listed in Table 5. The version and the supplier information are listed in the same table.

Table 5 Software packages

Software	Version	Company
Microsoft System software	XP professional	Microsoft Corporation
Labview	7.2	National Instruments
Excel 2002	SP-2	Microsoft Corporation
Word 2002	SP-2	Microsoft Corporation
Design Expert	6.0	Stat Ease Inc.

4.2 Methods

4.2.1 Solubility of Soy Protein Isolate

The solubility of the soy protein isolate was investigated at different pH's and in different buffers. The concentration of soy protein in each sample was 3.12% (wt/wt). The soy protein solution prepared in water was titrated to pH 11 with 10M NaOH. After taking a sample at a pH of 11, the pH was then titrated step by step to pH 9, 7, 5, 3 and 1.5. At each pH the solution was stirred for 2-3 minutes before a final sample was taken. The soy protein solutions in 0.1M NaP pH 7.8, 0.1M NaP pH 7.8 with 100mM NaCl, 0.1M Tris-HCl pH 7.8 and in 0.1M Tris-HCl pH 7.8 + 100mM NaCl were analyzed at the initial pH of 7.8. The concentration of protein was measured by Bradford. Prior to Bradford analysis, the samples were centrifuged for 5 minutes at 6'000g at RT. The supernatant was then diluted 1:50 (20µl sample + 980µL of Milli-Q-water) for use in the assay.

4.2.2 Bradford assay

For the Bradford assay a calibration curve with BSA was prepared. The concentrations used were 0 / 0.1 / 0.2 / 0.3 / 0.4 / 0.5mg/mL. 10µl of each sample, blank or standard were pipetted in triplicates in a microtiter plate. 200µl of diluted protein dye (1:5 dilution in water) was used for each well. After an incubation time of 5 minutes at RT the absorbance was read at 590nm with a microplate reader.

4.2.3 SDS-PAGE

1.5M Tris-HCl, pH 8.8

27.23g	Tris base
80ml	Milli-Q-water

The pH was adjusted to 8.8 with 10M HCl and the volume was brought to 150ml with Milli-Q-water. The solution was stored at 4°C.

0.5M Tris-HCl, pH 6.8

6g	Tris base
60ml	Milli-Q-water

The pH was adjusted to 6.8 with 10M HCl and the volume was brought to 100ml with Milli-Q-water. The solution was stored at 4°C.

2x Sample buffer (SDS Reducing Buffer)

3.55ml	Milli-Q-water
1.25ml	0.5M Tris-HCl, pH 6.8
2.5ml	Glycerol
2.0ml	10% SDS
0.2ml	0.5% bromophenol blue

Before use, 50µl of β-Mercaptoethanol was added to 1ml of the sample buffer.

1x Electrode Running buffer

3.03g	Tris base
14.4g	Glycine
1g	SDS

The components were dissolved and the volume was filled up to 1 L with Milli-Q-water. The pH was not adjusted. The solution was stored at 4°C.

10% APS

100mg of APS was dissolved in 1ml of Milli-Q-water.

Staining solution

160ml	MeOH
40ml	Acetic Acid
0.4g	Comassie blue

Filled up to 400ml with Milli-Q-water

De-staining solution

200ml	MeOH
35ml	Acetic Acid
265ml	Milli-Q-water

All components were mixed and stored at RT.

Sample preparation

SPI: 3µl of 10mg/ml soy protein isolate solution + 17µl Milli-Q-water

Enzymes: 3µl of 10mg/ml pepsin or pancreatin solution + 17µl of Milli-Q-water

Digestion: 5µl + 15µl of Milli-Q-water

Permeate of filtrated samples: 20µl

Every sample was then diluted 1:2 with 2x sample buffer followed by heating the samples for 4 minutes at 95°C.

15% Resolving gel

5ml	30% Acrylamide/Bis
2.5ml	1.5M Tris-HCl, pH 8.8
0.1ml	10% SDS
2.4ml	Milli-Q-water

Immediately prior to pouring the gel, the following components were added:

50µl	10% APS
5µl	TEMED

The mixture was then swirled gently to initiate polymerization and then poured into the gel chamber.

4% Stacking gel

1.3ml	30% Acrylamide/Bis
2.5ml	0.5M Tris-HCl, pH 6.8
0.1ml	10% SDS
6.1ml	Milli-Q-water

Immediately prior to pouring the gel, the following components were added:

50µl	10% APS
10µl	TEMED

The mixture was swirled gently to initiate polymerization and then poured into the gel chamber after (above) the polymerized 15% resolving gel.

The gel was placed into the running chamber, the samples loaded and then the voltage set to 100V. After 15 minutes the voltage was increased to 200V for another 30 minutes.

Afterwards the gel was taken out of the chamber and washed 3 times for 5 minutes in Milli-Q-water.

The gel was then stained over night with the staining solution.

The next morning the gel was destained with the destaining solution for about 2h and afterwards a digital picture was taken.

4.2.4 OPA assay

1mM Phenylglycine solution

0.015g of Phenylglycine powder was dissolved in 100ml Milli-Q-water.

1.5ml aliquots were stored at -20°C.

80mM Borax solution

14.81g of sodium borate * 10 H_2O was dissolved in 500mL Milli-Q-water.

10% SDS solution

50g of SDS powder was dissolved in 500ml Milli-Q-water

300mM o-phthaldialdehyde solution

0.08g of o-phthaldialdehyde powder was dissolved in 2ml 95% Ethanol

OPA solution

50ml	80mM Borax solution
20ml	10% SDS
2ml	300mM o-phthaldialdehyde solution
200µl	β-Mercaptoethanol

The mixture was topped up to 100ml with Milli-Q-water and the pH was adjusted to 9 with 1M HCl.

Phenylglycine standard curve

For the standard curve, 5 different concentrations of phenylglycine were prepared. The dilution of the 1mM solution is described in table 6.

Table 6 Amount of water and phenylglycine (phegly) solution necessary for the OPA assay standard curve

Sample Concentration [µM]	Milli-Q-water [µl]	1mM Phegly solution [µl]
0	1000	0
250	750	250
500	500	500
750	250	750
1000	0	1000

100µL of each standard was placed into a 1.5ml cuvette (1:10 dilution). For the digestion samples only 10µl was taken for the analysis. For the samples taken at the end of the digestion a dilution of 1:5 was prepared and of that dilution 10µl was taken.

To ensure accuracy it was necessary to give each sample an incubation time of 2 minutes before each spectrometer reading. Measurements were taken at time intervals according to Table 7. Because only 5 samples could be read at any one moment, samples were usually carried out in quadruplicates. The absorbance was measured at a wavelength of 340nm.

Table 7 OPA assay timetable

Time [s]	Action
0	Place 1ml of OPA solution in 0µM cuvette
20	Place 1ml of OPA solution in 250µM cuvette
40	Place 1ml of OPA solution in 500µM cuvette
60	Place 1ml of OPA solution in 750µM cuvette
80	Place 1ml of OPA solution in 1000µM cuvette
100	Read absorbance of 0µM sample
120	Read absorbance of 250µM sample
140	Read absorbance of 500µM sample
160	Read absorbance of 750µM sample
180	Read absorbance of 1000µM sample

4.2.5 Enzymatic digestions of Soy Protein Isolate

3.12% (w/w) SPI solution

A mass of 4.99g of soy protein isolate was dissolved in 160ml of Milli-Q-water and was stirred for several minutes until all parts were well dissolved. The pH was adjusted to the desired pH with 10M/1M HCl or 10M/1M NaOH. (For Pepsin digestion to pH 1.5 and for Pancreatin digestion to pH 7.8)

0.1M NaP pH 7.8

2.2g of Na_2HPO_4 was dissolved in 400ml Milli-Q-water and the pH was set to 7.8 with 10M/1M NaOH. Filled up to 500ml with Milli-Q-water.

0.01M HCl

10ml 0.1M NaCl + 90ml of Milli-Q-water.

0.005g/L Pancreatin solution (final concentration in digestion solution 8mg/L)
0.15g Pancreatin was dissolved in 30ml 0.1M NaP pH 7.8 for a digestion of 150ml of 3.12% (w/w) soy protein isolate.

0.005g/L Pepsin solution (final concentration in digestion solution 0.25mg/L)
0.0375g Pepsin was dissolved in 7.5ml 0.01M HCl for a digestion of 150ml of 3.12% (w/w) soy protein isolate.

150mM Sodium carbonate
1.6g of sodium carbonate was dissolved in 100ml of Milli-Q-water

During each digestion, a control was run with 10ml SPI in a falcon tube with no addition of enzymes. The temperature of the water bath was set at 37°C for the pepsin digestion and the SPI solution was preheated. At Time 0 minutes a sample of 10µl of the control and 4x 10µl for the digestion was taken to measure the concentration of peptides by OPA assay (See OPA assay procedure for details). The timer was started after adding the Pepsin to the SPI solution. 10µl of samples at Time 15 and 30 minutes was taken and the peptide concentration measured. If the digestion was to not continue with pancreatin the pH was titrated to 7 to inactivate the enzyme and the sample was frozen at -20°C. If the digestion was to continue with pancreatin the following steps were carried out. The temperature for the water bath was set at 40°C for the pancreatin reaction, the SPI solution was titrated to a pH of 7.8 with 10M/1M NaOH, and the solution was allowed to warm up in the water-bath. Once the sample temperature reached 40°C pancreatin was added to the solution and the timer was started. Samples were taken after 15 / 30 and 60 minutes for the measurement of the peptide concentration. After 60 minutes the addition of 1050µl of 150mM sodium carbonate to the 150ml digestion was performed to stop the reaction. (Final concentration of 0.8mM) The sample was then frozen at -20°C, or in some cases the samples were used for the filtration step.

4.2.6 Freeze drying of samples

A known volume of a sample (mainly 30ml) was frozen at -20°C over night in a 50ml falcon tube (not more than 30ml). The next day the samples were frozen in liquid nitrogen at -196°C. Afterwards the falcon tubes were freeze dried in the freeze dryer for 3 days under pressure of 1mbar and a temperature of -40°C.

4.2.7 Dead end filtration

10% Ethanol solution

10ml of 98% Ethanol + 90ml of Milli-Q-water

The membrane was first soaked for 1.5h in Milli-Q-water to remove the glycerine (which is used prevent the membrane from drying during storage). Milli-Q-water was replenished 3 times during the 1.5h period. Afterwards the system could be assembled as seen in Figure 3.

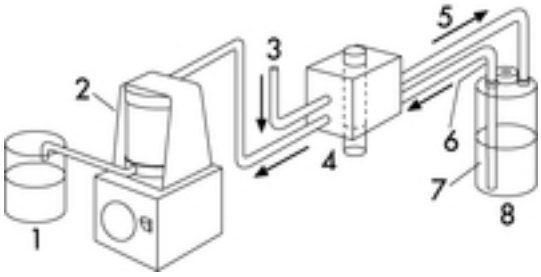

Figure 3 Millipore Amicon filtration unit (1-, 2-, 3-, 4-, 5-, 6-, 7-, 8-)

The parameters of the Amicon stirred cell are listed in table 8.

Table 8 Millipore membranes and Amicon 8200 stirred cell parameters.

Parameter	
Maximum process volume	200mL
Minimum process volume	5mL
Membrane diameter	63.5mm
Effective membrane area	28.7mm^2
Hold-up volume	1.2mL
Expected YM 1 Waterflux (at 55 psi)	12-24 L/(m^2*h)
Expected YM 1 Solute flux (at 55 psi)	18 L/(m^2*h)
Expected YM 3 Waterflux (at 55 psi)	36-48 L/(m^2*h)
Expected YM 3 Solute flux (at 55 psi)	42 L/(m^2*h)

Before the filtration could be started a water flux of the membrane was measured at 10 / 20 / 30 / 40 and 50 psi. A balance was used to measure the mass of the permeate.

The exact amount of the feed solution was determined before starting the filtration. The filtration was run till approximately 50ml of permeate was received. After the filtration, the membrane was stored in a Petri-dish in 10% Ethanol at 4°C. Filtration data was collected with the Labview 7.2 software and later evaluated in Excel.

To achieve a mass-balance, the peptide content in the permeate and the retentate were measured with the OPA assay, as well as a total solids established (see section 1.2.8). For the total solids membrane weight was accounted for through a drying process.

4.2.8 Total solids

The total solids were measured in the retentate and the permeate after a filtration. A volume of 1mL of sample was added to a pre-weighed glass tube. The glass tubes were placed over night in the oven at 100°C. The next day the glass tubes were placed in a desiccator to allow cooling. The tubes were then weighed again and the concentration of total solids in the samples could be calculated.

4.2.9 DPPH assay

0.2M MES solution

A volume of 1ml 1MES buffer was combined with 4ml of Milli-Q-water.

1mM Ascorbic acid (was prepared freshly every day)

A mass of 0.0176g powder was dissolved in 100ml of Milli-Q-water.

Ascorbic acid standard curve

The standard curve was prepared with 5 different concentration of ascorbic acid. Table 9 lists the dilutions used for the standard curve.

Table 9 Amount of water and ascorbic acid necessary for DPPH the standard curve

Sample Concentration [mM]	Milli-Q-water [µl]	1mM ascorbic acid [µl]
0	1000	0
0.2	800	200
0.3	700	300
0.4	600	400
0.5	500	500

DPPH solution

A mass of 0.008g of DPPH (400µM) was dissolved in 25ml 98% Ethanol and was mixed until no traces of powder were visible. A volume of 20ml of Milli-Q-water and 5ml of 1M MES buffer were added. pH was titrated to 6 with 10M NaOH. The solution was then stored in a brown glass bottle and wrapped with aluminum foil.

Sample preparation

300µl sample or standard + 300µl 0.2M MES + 600µl 98% Ethanol + 1200µl DPPH solution in a 4ml cuvette.

The spectrometer is blanked with 98% Ethanol as a control; for the DPPH solution; a combination of 1200µl DPPH and 1200µl 98% Ethanol was used.

After pipetting the DPPH solution to the samples, the cuvettes were placed as fast as possible into the spectrometer. The absorbance at a wavelength of 517nm was monitored every minute for a 20 minutes period.

4.2.10 MALDI-TOF analysis

The digested samples were freeze dried for the analysis. 10mg of each sample was sent to the University of Guelph for analysis with the MALDI-TOF. A spectrum from 500 to 4000kDa was performed.

Prepared samples:
1: Soy protein isolate undigested
2: Soy protein isolate digested with pepsin (30min; 37°C; pH 1.5)
3: Soy protein isolate digested with pancreatin (60min; 40°C; pH 7.8)
4: Soy protein isolate digested with pepsin & pancreatin (30min; 37°C; pH 1.5 and 60min; 40°C; pH 7.8)

4.2.11 TGA analysis

The thermal gravimetric analysis was carried out between of 40 to 650 °C.

4.2.12 Experimental design

A 2^4 level factorial design was used to study the relationship and influence of 4 digestion variables on the final digestion peptide concentration. The 4 factors studied were:

A: Final conc. of pepsin; **B**: Final conc. of pancreatin; **C**: time of pepsin; **D**: time of pancreatin

For each factor two levels were used, a high and a low level. To gain a better understanding of experimental variation three centre point experiments were also performed. Table 10 displays the high and low levels for each independent experimental factor.

Table 10 High and low levels for each experimental parameter investigated in the factorial design. The concentrations are presented as final concentration in the digestion solution.

Factor	Low Level	Centre point	High Level
A	0.149 mg/L	0.223 mg/L	0.297 mg/L
B	0.5 mg/L	1.5 mg/L	2.5 mg/L
C	15 min	30 min	45 min
D	60 min	90 min	120 min

The experimental design was created with the Design Expert 6.0 software. A randomization of the run order was performed to minimise possible random latent variable effects such as time of day, system readings etc.

Table 11 Experimental design summary. High levels (1), centre points (0), low levels (-1).

Run	Factor A	Factor B	Factor C	Factor D
7	-1	-1	1	1
8	-1	-1	-1	-1
12	1	-1	1	1
13	1	-1	1	-1
15	-1	-1	1	-1
16	1	-1	-1	-1
17	-1	-1	-1	1
18	1	-1	-1	1
9	0	0	0	0
10	0	0	0	0
11	0	0	0	0
1	1	1	1	1
2	-1	1	1	-1
3	1	1	-1	1
4	-1	1	-1	-1
5	-1	1	1	1
6	-1	1	-1	1
14	1	1	1	-1
19	1	1	-1	-1

The digestions were carried out as described in part 1.2.5 (enzymatic digestion of soy protein isolate) at a 150ml scale. Samples were taken at different time points for the different levels:

Low pepsin time: 0 and 15 minutes

High pepsin time: 0 / 15 and 45 minutes

Low pancreatin time: 15 / 30 and 60 minutes

High pancreatin time: 30 / 60 and 120 minutes

The samples at each time point were analyzed by the OPA assay at a wavelength of 340nm. With increasing digestion time the samples required a 5x dilution for the OPA assay. A 10µl of this dilution was taken for the OPA assay. After digestion completion, each sample was frozen at -20°C for further analysis and/or filtration.

The concentration of peptides at the very end of the digestion was taken for statistical evaluation. With the + + + +; centre point and - - - - sample, a filtration with the 3kDa and the 1kDa membrane was carried out, as described in part 1.2.7. The retentate and the permeate of each filtration were analysed by OPA assay and by total solids estimation. The permeate of 3kDa membrane and the retentate and the permeate of the 1kDa membrane were also analyzed by the DPPH assay to measure the antioxidative properties.

All digestion samples were loaded onto a 15% SDS-PAGE. The samples were prepared as follows:

Sample: 5µl digestion + 15µl milli-Q-water + 20µl sample buffer
SPI: 3µl 10mg/ml SPI + 17µl milli-Q-water + 20µl sample buffer
Broad range marker: 10µl marker + 10µl sample buffer

→ Description of the SDS method can be see in part 1.2.3

5 Results

5.1 Digestion of soy protein isolate from ADM

5.1.1 Solubility of SPI

The solubility of soy protein in aqueous solution was estimated at different pH (1.5 / 3 / 5 / 7 / 9 / 11) and in different buffers (H_2O, NaP, Tris, NaCl). At the different conditions, a sample was taken, centrifuged and analysed by Bradford to investigate the dissolved protein concentration. The experiment was repeated 3 times. Figure 4 proves that the solubility around the isoelectric point of the protein is very low. If the pH is increasing or decreasing from the isoelectric point the solubility is increasing again. According to Figure 4 the highest solubility is at a basic pH of 11 with 47%. The digestions were performed at a pH of 1.5 and 7.8 and correspond to a similar solubility of approximately 38% of the soy protein content estimated by Bradford. The comparison of the different solvents shows that the addition of salt decreases the solubility of the soy protein. If there is an additional addition of sodium chloride in the solvent the solubility is decreasing further.

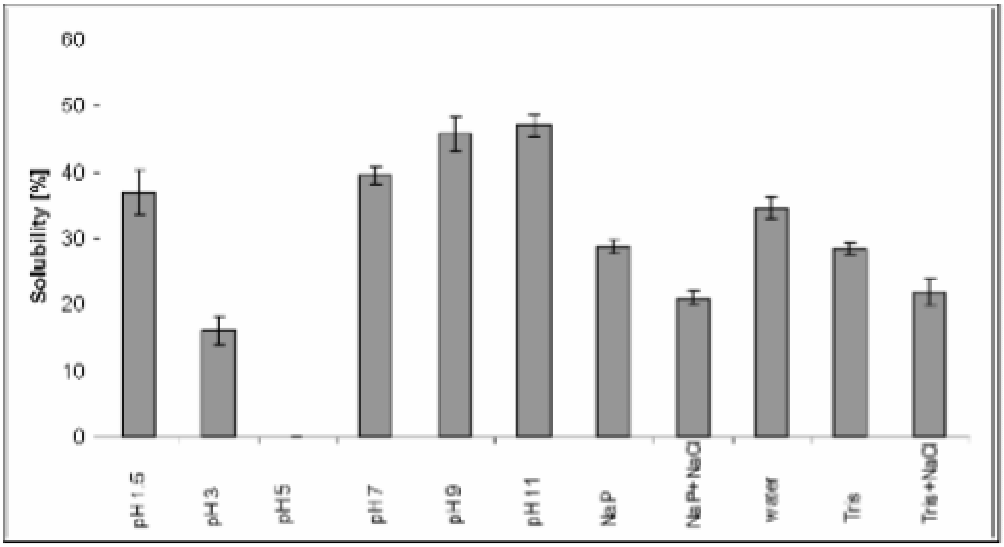

Figure 4 Profile of the solubility of soy protein (different pH and different buffers). (n=3)

5.1.2 Comparison of different digestion enzymes

The initial method of the digestion of 3.12% w/w soy protein isolate (SPI) PRO FAM® 974 from ADM was a 30 minute digestion with 0.25 mg/L pepsin at pH 1.5 and 37°C, followed by a digestion for 60 min with 8 mg/L pancreatin at a pH of 7.8 and 40°C. To see the influence of each enzyme, the SPI was digested with either pepsin only or with pancreatin only. Each digestion (pepsin only / pancreatin only / pepsin & pancreatin) was carried out in triplicates and each point measured in quadruplicates. According to the results presented in Figure 5 a standard deviation lower than 10% was achieved for each measuring point, proving a good reproducibility is attained.

As shown in Figure 5 the digestion with pancreatin only or pepsin only is leading to about the same concentration of peptides at about 20mM (calculated as equivalent Phe-Gly), however one should keep in mind that the incubation time of pancreatin is double that of pepsin. It is only when a combination of both enzymes is used, that the peptide concentration increases to over 30mM.

A control without any addition of enzymes was completed for each digestion experiment, to see if the concentration of peptides is changing during the time at an increased temperature. According to Figure 5 the amount of peptides is stable during 90 minutes at around 12mM.

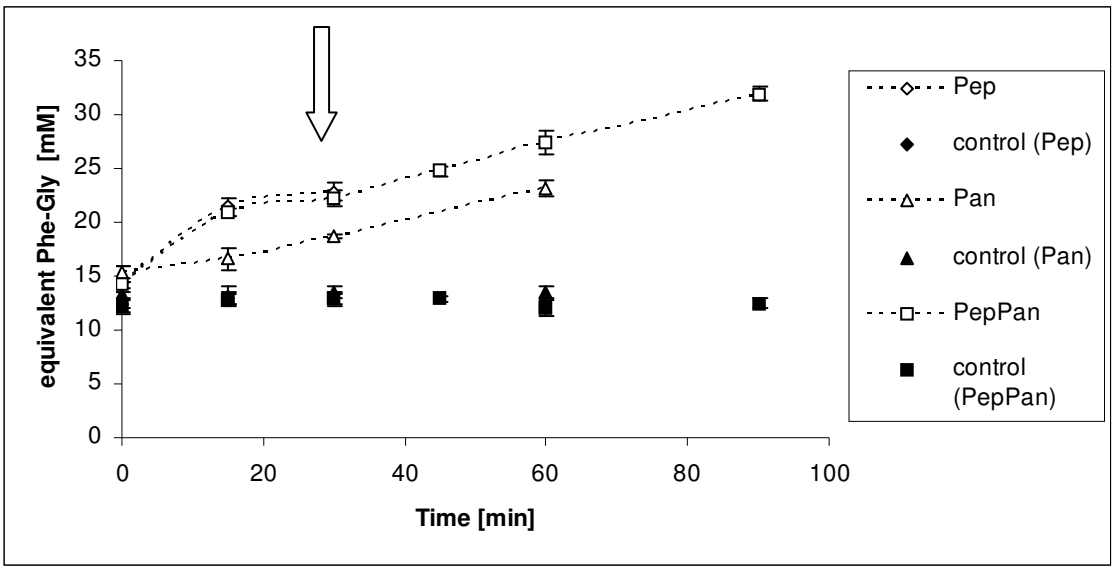

Figure 5 Peptide concentration (equivalent Phe-Gly) during different digestions with pepsin only, pancreatin only and pepsin & pancreatin as a function of time (n=3). The arrow shows the changing of the enzyme.

5.1.3 Influence of pH and SPI concentration for the Pepsin digestion

The influence of a pH shift and a higher concentration of soy protein isolate during the pepsin digestion were investigated. A pH of 1.5 (original) / 2 and 2.5 and a SPI concentration of 3.12% w/w (original) and 5% w/w were compared. The digestion time of 30 minutes and the temperature at 37°C were not changed.

According to Figure 6, the pH doesn't have a significant effect on the production of peptides (calculated as equivalent Phe-Gly) for the pH range investigated. An increase in SPI concentration by a factor of 1.5 resulted in a corresponding increase in peptide concentration. This proves that the enzyme is not limiting the reaction.

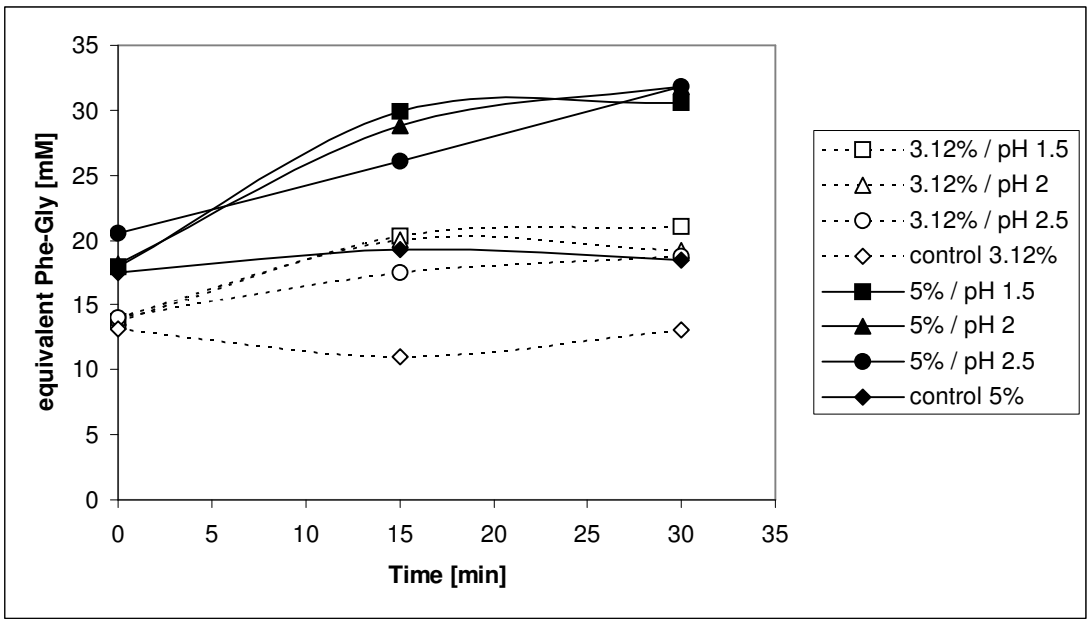

Figure 6 Effect of SPI concentration and pH on the peptide yield during a pepsin digestion.

5.2 Digestion of soy protein isolate from Solae

5.2.1 Comparison of ADM and Solae SPI digestions

With the soy protein isolate from ADM, that was used originally, a TGA analysis was performed. Because the profile of this TGA was very different in comparison to the soy protein isolate from Solae, a digestion with pepsin & pancreatin with the SPI from Solae was performed to see if there would be a difference between the two substrates.

According to Figure 7, the SPI of Solae yields a higher peptide concentration (calculated as equivalent Phe-Gly) for the entire digestion. If the initial peptide concentration is taken into account, the Solae SPI resulted in a 6.4mM higher peptide concentration. The initial digestion with pepsin had a similar profile for both SPI. The digestion with pancreatin had a higher

slope just after the addition of pancreatin, what led to a higher yield of peptides after 90 minutes.

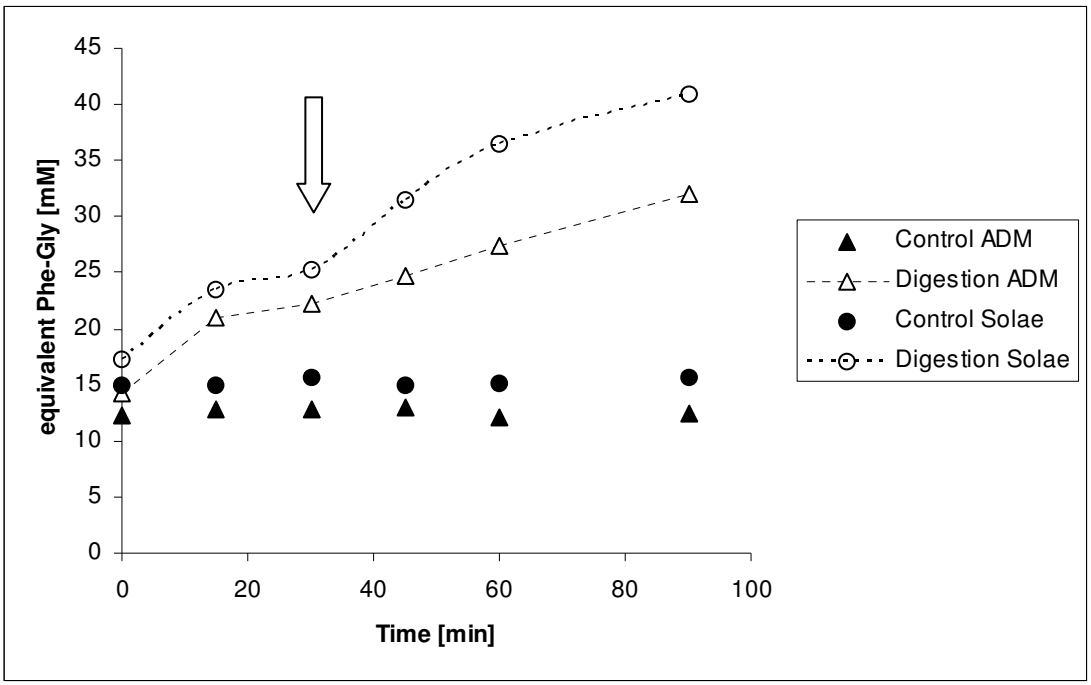

Figure 7 The production of peptides (in equivalent Phe-Gly) during a digestion with pepsin (initial 30 min) & pancreatin (subsequent 60 min) as a function of time for two different types of soy protein isolate. The arrow shows the changing of the enzyme.

5.2.2 Filtration of ADM and Solae SPI Hydrolysates

The two digestions with pepsin & pancreatin, performed with two different soy protein isolates (ADM and Solae) were filtered with stirring using a 3kDa and a subsequent 1kDa membrane to see the effect of the different substrates.

According to Figure 8, both SPI hydrolysates with the 3kDa membrane resulted in approximately the same permeate flux versus time profile. The 1kDa filtration with the digested solution from ADM had a higher flux of 14L/(m^2*h) than the digested solution from Solae with a flux of only 8L/(m^2*h).

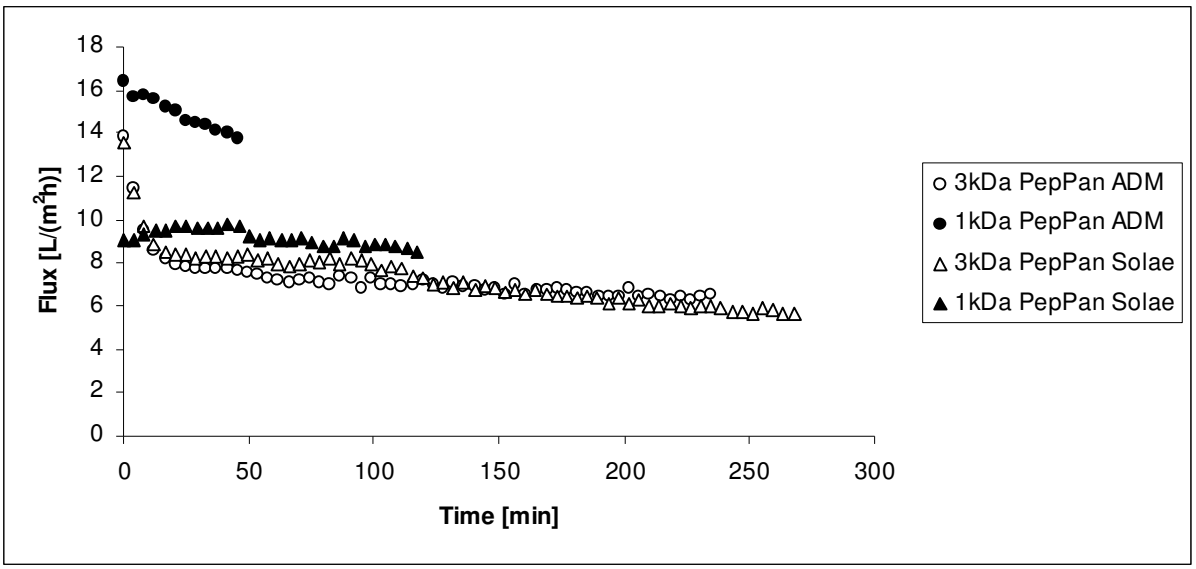

Figure 8 The flux as a function of time for the dead-end ultrafiltration with a 3kDa and a 1kDa membrane dead-end filtration of a digested soy protein isolate sample (with pepsin & pancreatin) from ADM and Solae

5.2.3 Mass balance during a filtration

From the filtration of the SPI (from Solae) with the combined pepsin & pancreatin digestion a mass balance for the peptide concentration and for the total solids was performed to see the distribution of the peptides during the 3kDa and the 1kDa filtration.

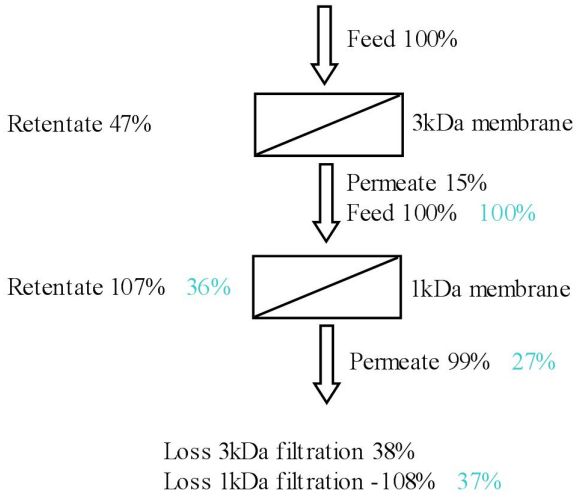

Figure 9 Massbalance of the 3kDa and the 1kDa filtration of the Solae hydrolysates with the peptides in black and the total solids results in **turquoise**

The determination of the total solid content was only performed for the 1kDa filtration. After the 1kDa filtration, 36% of the total solids content was recovered in the retentate and 27% was in the permeate. The distribution of the total solids is not similar with the distribution of the peptides.

According to Figure 9, approximately half of the peptides are recovered in the retentate of the 3kDa membrane, which means that these peptides are larger than 3kDa or the pores of the membrane are blocked. Only 15% of the peptides were recovered in the permeate of the 3kDa filtration. During the 3kDa filtration 39% of the peptides were lost. This amount of peptides could be contributing to pore blockage or could be part of the cake.

Based on the peptide mass balance, peptides were produced during the 1kDa filtration which is not realistic. However, a mass balance based on total solids the total solids show lower values after the filtration.

5.3 Filtration of SPI hydrolysates from ADM

5.3.1 Comparison of different digestion methods for SPI from ADM

The digestions, that were carried out with pepsin only (30min digestion → peptide concentration: 20mM), pancreatin only (60 min digestion → peptide concentration: 20mM)) and with pepsin & pancreatin (90min digestion → peptide concentration: 30mM) were filtered with a dead-end system through a 3kDa and a subsequent 1kDa membrane. The system was used in stirring mode. According to Figure 10, the 1kDa membrane permeate flux of the filtrations are quite similar for all digestion conditions investigated. The permeate flux profile for the 3kDa membrane filtrations had quite different profiles according to the digestion conditions.

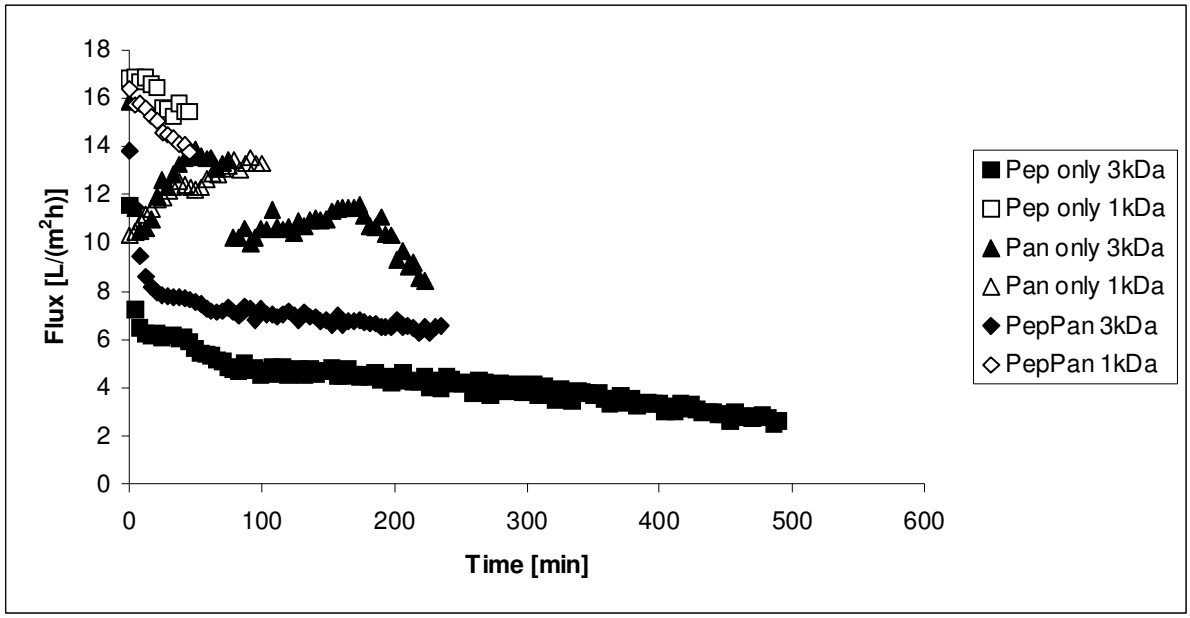

Figure 10 The permeate flux as a function of time for a dead-end filtration with stirring of pepsin only, pancreatin only, and pepsin & pancreatin digestions of the ADM SPI

In Figure 11 a better differentiation of the 6 different filtrations of the (pepsin only / pancreatin only and pepsin & pancreatin digestions with the 3kDa and 1kDa membrane) is presented. The permeate volume as a function of time shown for all 1kDa filtrations have approximately the same profile. The pancreatin only digestion resulted in the highest slope for the 3kDa filtration, meaning the flux was the highest. The pepsin only digestion had the lowest slope, and the slope of the pepsin & pancreatin digestion was found between the two extremes.

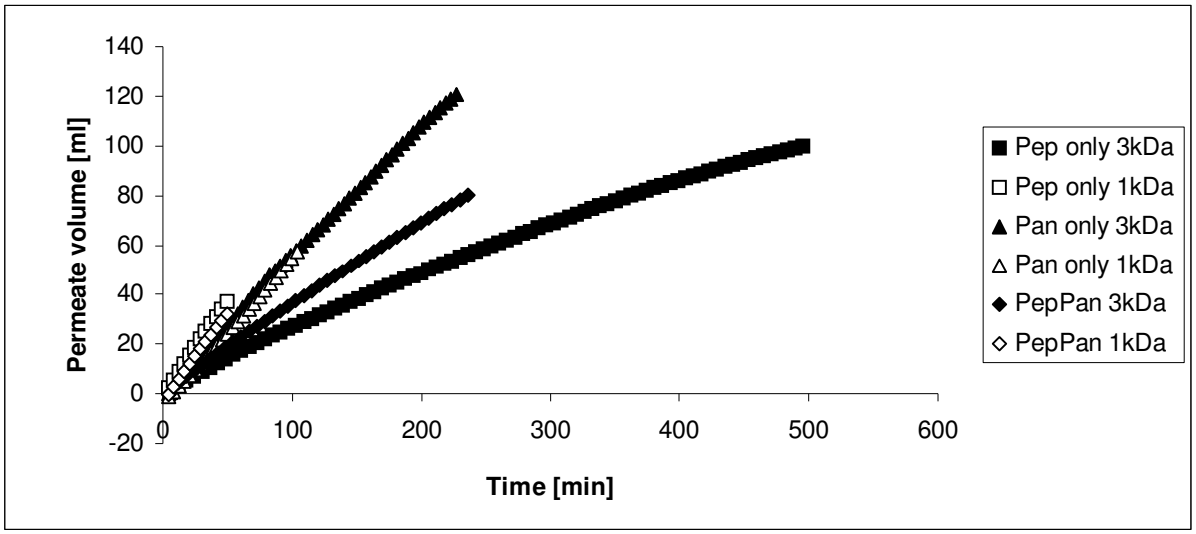

Figure 11 The volume of the permeate as a function of time for a dead-end filtration with stirring of pepsin only, pancreatin only, and pepsin & pancreatin digestions of the ADM SPI

5.4 Characterisation of soy protein isolate

5.4.1 TGA profile of SPI

The thermal degradation and the ash content of two different soy protein isolates was analyzed by TGA (Thermal Gravimetric Analysis) and is illustrated in Figures 12 + 13. The degradation was followed from 40 till 650°C with a heating rate of 0.5°C/min. The degradation of both SPI starts at ~ 250 °C. The decomposition can be described by the derivative of the received curve (weight in function of temperature). Each peak is then associated with one particular type of degradation. The peak in both figures at around 90°C comes from the water. The analysed ADM PRO FAM SPI sample shows only one peak. In the Solae SPI sample, two peaks are observed. These two peaks are most likely the glycinin and the β-conglycinin, the major proteins of SPI. The ash content, the weight remaining after 650 °C, of the ADM sample was 24% and 6% for the Solae sample. Therefore the two samples also have different ash content.

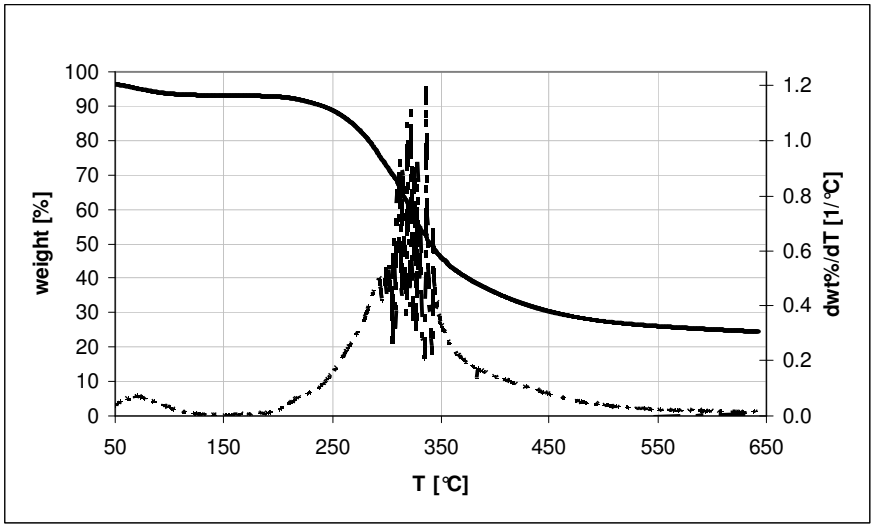

Figure 12 Thermal degradation profile by TGA of the ADM PRO FAM 974 SPI

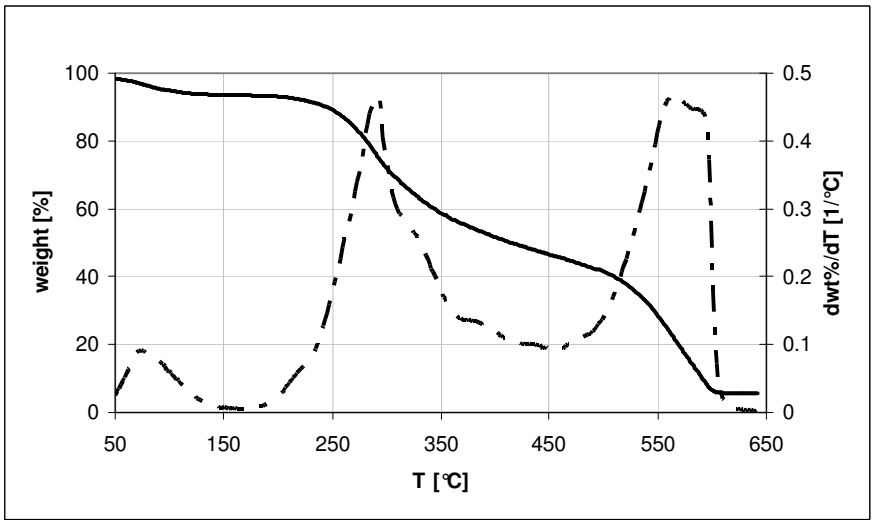

Figure 13 Thermal degradation profile by TGA of the Solae SPI

5.4.2 Peptide analysis by MALDI-TOF

To investigate the size distribution of the peptides produced during a digestion of SPI, the MALDI-TOF method was used. A sample of the undigested soy protein isolate, a digestion with pepsin only, a digestion with pancreatin only and a digestion with pepsin & pancreatin were prepared and freeze dried. The freeze dried samples were sent to the University of Guelph for the analysis. The range of the molecular weights analyzed was between 500 and 4000 Da.

According to Figure 14 the undigested soy protein isolate sample shows peptides in the molecular weight range between 500 and 1500Da. A digestion with pepsin only is producing several peptides between the molecular weight range of 500 and 4000 Da. The digestion with pancreatin only isn't that effective for the production of peptides between a mass range of 2000 to 4000 Da. According to Figure 16, only the peptides between a size of 500 and 2000 Da are produced. It needs to be assumed that bigger peptides are produced during the digestion, as approximately the same amount of peptides should be produced during a digestion with pepsin only and pancreatin only. The Figure 17 shows that a large amount of peptides between the molecular weight range between 500 and 4000 Da are produced during a digestion with pepsin and pancreatin. Even below 500 Da a number of peaks are visible, indicating that a number of the peptides produced are smaller 500Da.

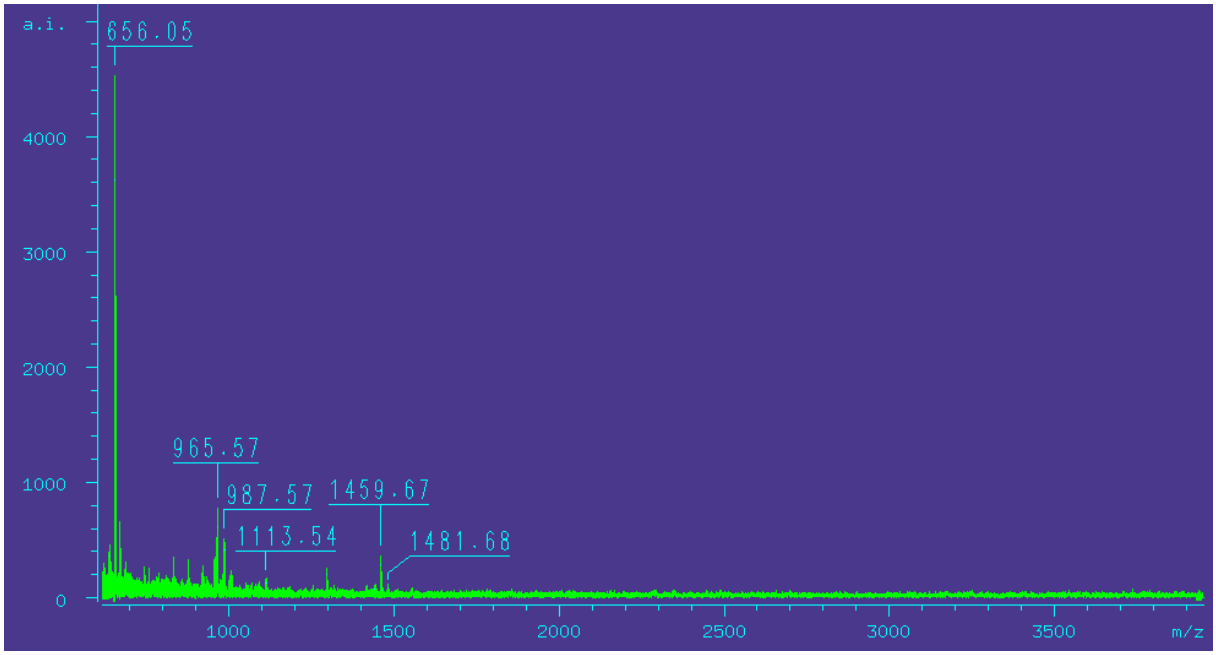

Figure 14 Undigested soy protein isolate (ADM) sample analysed by MALDI-TOF with a molecular range between 500 and 4000 Da

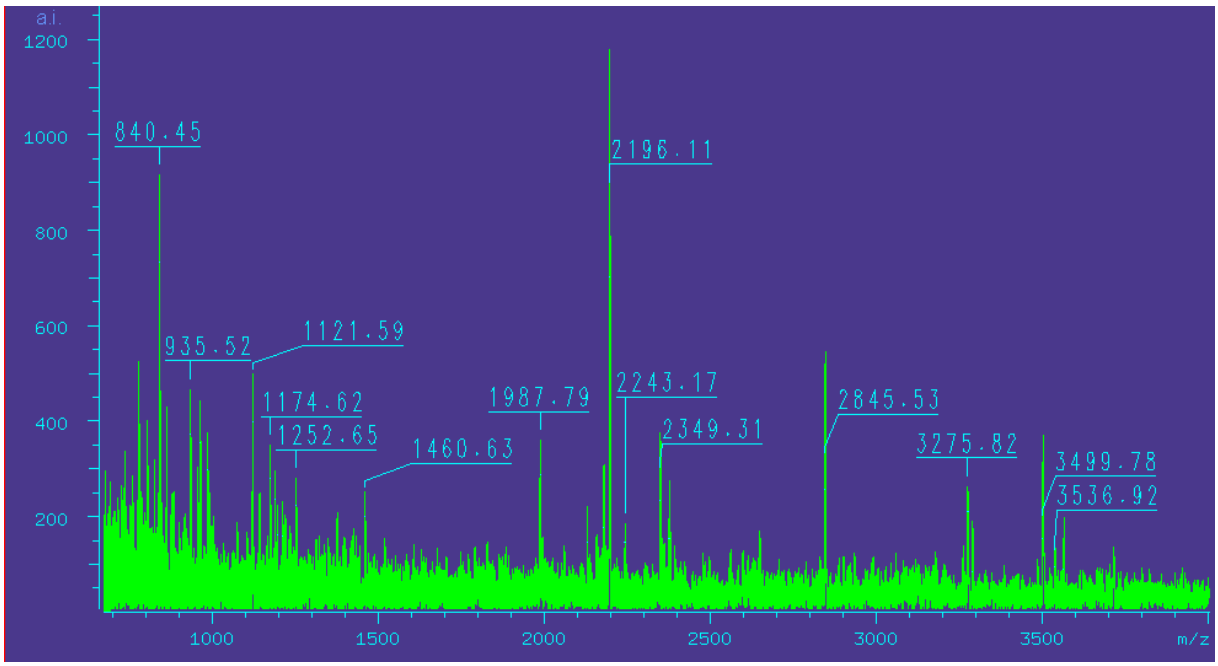

Figure 15 Soy protein isolate (ADM) sample digested with pepsin only analysed by MALDI-TOF with a molecular range between 500 and 4000 Da

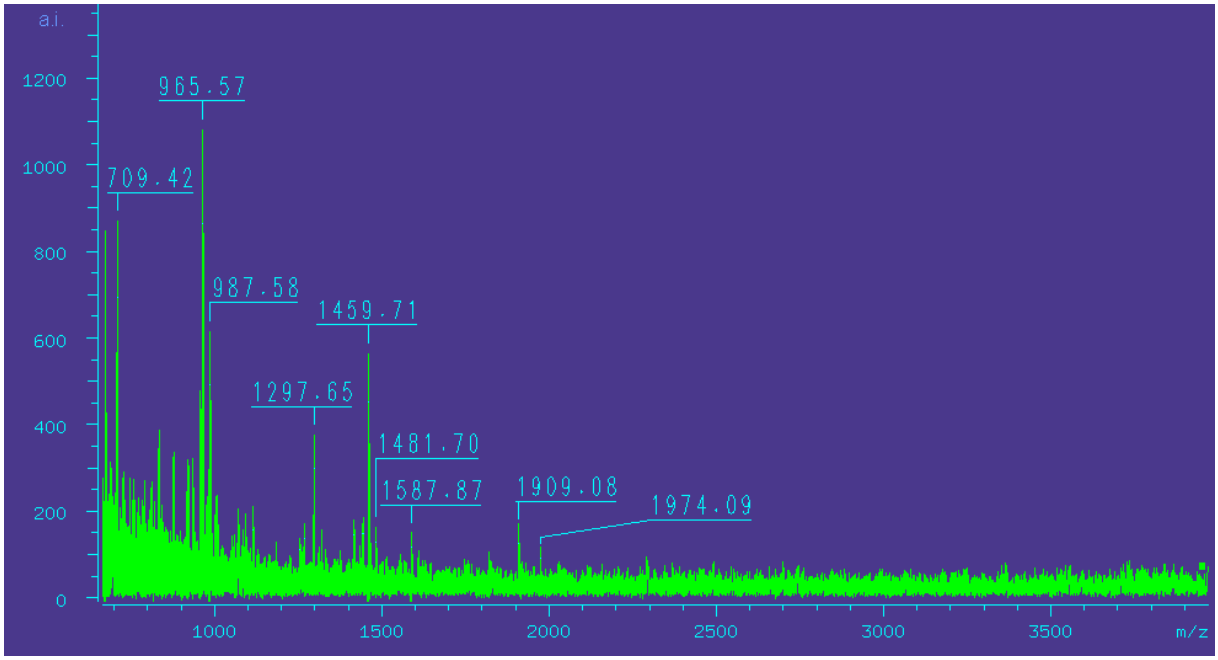

Figure 16 Soy protein isolate (ADM) sample digested with pancreatin only analysed by MALDI-TOF with a molecular range between 500 and 4000 Da

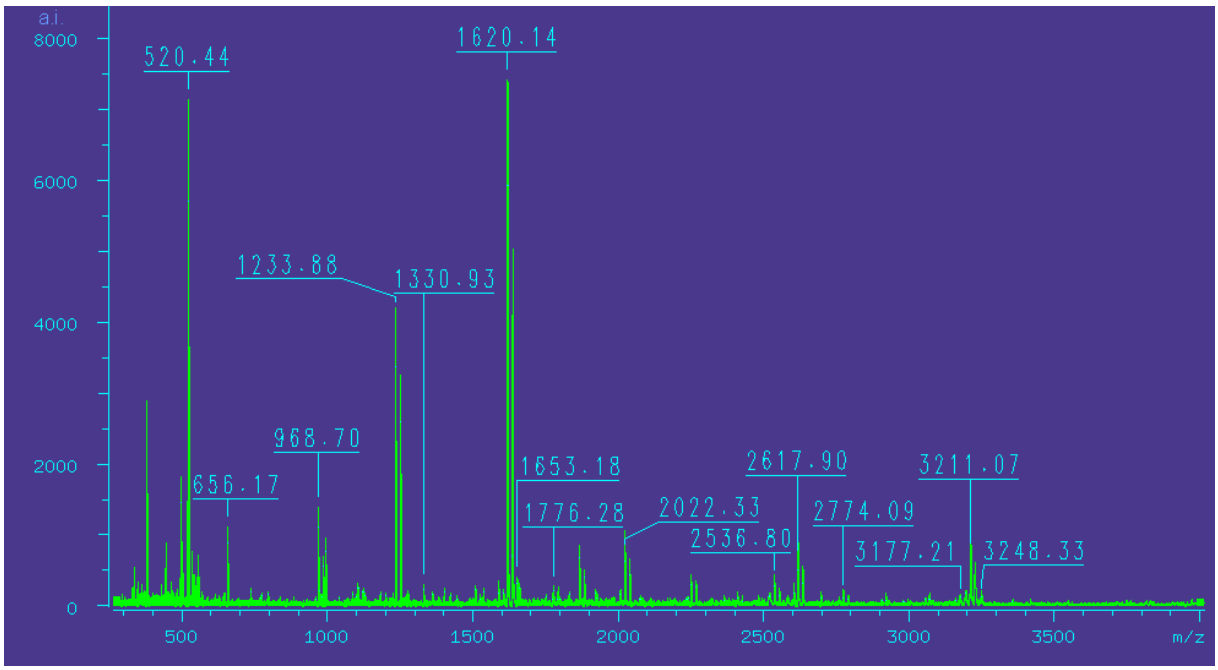

Figure 17 Soy protein isolate (ADM) sample digested with pepsin & pancreatin, analysed by MALDI-TOF with a molecular range between 500 and 4000 Da

5.4.3 Peptide analysis by SDS-PAGE

The molecular weights of different samples were investigated by SDS-PAGE. In this method charge on all molecules is the same due to SDS addition, therefore the molecules are separated according to their size. The molecular weight markers could be used as a calibration curve (logarithms of the known molecular weight of the protein vs. mobility) and the molecular weights of the other samples could be determined.

Because soy protein isolate contains mainly the two globulins, glycinin and β-conglycinin with different subunits and polypeptides, different bands were expected. The molecules with sizes of 42kDa and 14kDa, evaluated by SDS-PAGE could be related to the two chains of glycinin. [5] For the different subunits of β-conglycinin the molecular weights of 74kDa, 58kDa, and 47kDa could be related. [5] These molecular weights are similar to the theoretical value. The polypeptide with a size of 25kDa can be related to the trypsin inhibitor. [24]
The pepsin has a theoretical molecular weight of 34.6kDa. [15] Because the protein structure includes three disulfide bonds, four bands smaller than 34.6kDa would be expected after the treatment with β-mercaptoethanol in the sample buffer. The higher molecular weight bands might be impurities. The pancreatin is a blend of enzymes, therefore more bands were expected. [16]

The digestion with pepsin only or pancreatin only show a large number of high molecular weight polypeptides, in comparison to the digestion with both enzymes that shows only a broad smear around 10kDa. According to the different bands remaining for the pepsin only or the pancreatin only digestion, the pepsin causes a higher digestion of the globulin β-conglycinin than the pancreatin.

According to Figure 18, there are no visible bands in the permeate of the filtrations. It seems that the concentration of peptides is very low and therefore undetectable.

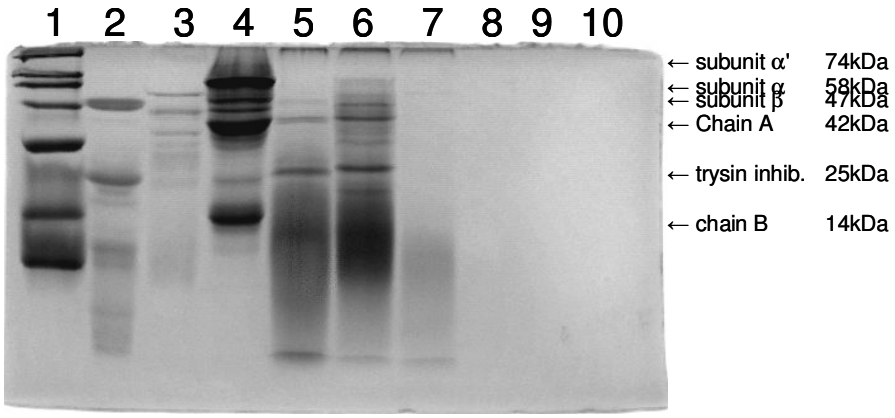

Figure 18 Polypeptide profile for undigested and digested soy protein isolate (ADM) by a 15% SDS-PAGE, stained with coomassie blue. On the right side of the gel there are listed the molecular weights of the SPI sample from the different chains and subunits.

Lane **1:** Marker; **2:** Pepsin; **3:** Pancreatin; **4:** SPI; **5:** Digestion with Pepsin only (30min, pH 1.5, 37°C); **6:** Digestion with Pancreatin only (60min, pH 7.8, 40°C); **7:** Digestion with Pepsin & Pancreatin (30min at pH 1.5 and 37°C; 60min at pH 7.8 and 40°C); **8:** YM3 Permeate of a Pancreatin only filtration; **9:** YM1 Permeate of a Pancreatin only filtration; **10:** empty

5.5 2^4 Factorial design to investigate digestion

5.5.1 Digestions

For the factorial design included 16 experiments runs with an additional 3 centre points. The centre points showed a good standard deviation of 6%. Table 12 shows that with a high level of pancreatin (Factor B) the peptide concentration after digestion is visibly larger than the peptide concentrations after low pancreatin level digestions.

Table 12 Factorial design conditions for the pepsin-pancreatin digestion of ADM SPI. The experiments are sorted by increasing peptide concentration. The 1 shows high level and the -1 shows a low level used for the experiments.,

Pep conc.	Pan conc.	Pep time	Pan time	Equivalent Phe-Gly [mM]	Run
1	-1	-1	-1	25.91	16
-1	-1	-1	-1	27.49	8
1	-1	1	-1	30.41	13
-1	-1	-1	1	30.97	17
-1	-1	1	-1	32.08	15
1	-1	1	1	33.61	12
1	-1	-1	1	34.74	18
-1	-1	1	1	36.75	7
0	0	0	0	38	10
0	0	0	0	41.62	9
0	0	0	0	42.6	11
1	1	-1	-1	44.37	19
-1	1	-1	-1	45.5	4
1	1	1	-1	45.85	14
-1	1	-1	1	46.76	6
-1	1	1	-1	47	2
1	1	-1	1	47.28	3
1	1	1	1	48.14	1
-1	1	1	1	48.47	5

The results from Table 12 (shown in mM equivalent phenylglycine) were inserted into a statistical software package (Design Expert 6.0). Figure 19 shows a half normal plot for the factorial design data. Factors outside the predominant linear curve are chosen as factors with a significant influence on final peptide concentration. The factors B (pancreatin concentration), D (time of pancreatin) and C (time of pepsin) with the blue point were chosen as effect factors. The factor that has the biggest distance to the linear curve has the most influence on the resulting model (in that case it is factor B, then D, then C). From this plot no interaction terms (AB, AC etc.) were deemed to have a significant effect on peptide concentration. The green triangles represent the centre points.

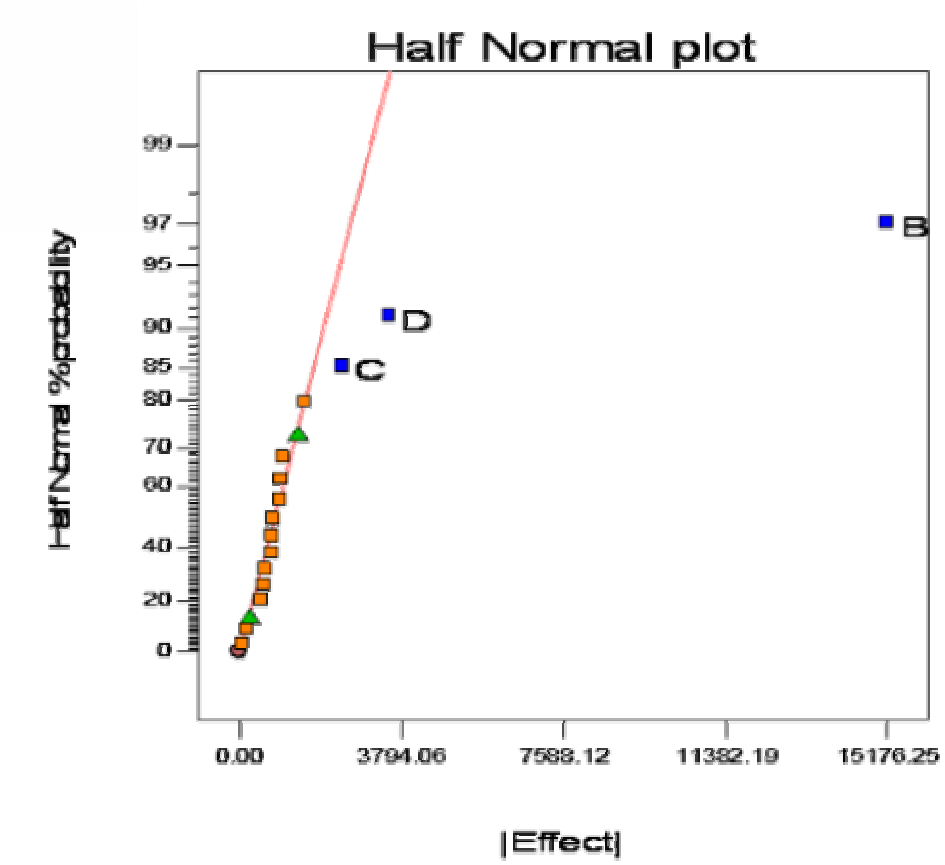

Figure 19 The half normal plot of the factorial design that shows the factors which have an effect on the system, in that case the blue points are the influencing factors (B (pancreatin concentration), C (time of pepsin), D (time of pancreatin))

An equation to describe the effect of the significant experimental factors on final peptide concentration was calculated as:

Peptide concentration = 39.08 + 7.59*B + 1.21*C + 1.76*D (1)

Subtracting the predicted values (equation 1) from the actual values gives a measure of the residual for any 1 experimental value. The plot in Figure 20 shows the studentized residuals vs. the predicted values. If these values are randomly distributed on the chart no data transformation (such as a logarithmic transformation) is needed for further analysis. As well if any of the studentized residuals would be found to be above 3.00, that point would be considered an outlier and removal from the model should be considered. In this case, the residuals were randomly distributed and no point had a studentized residual greater than 1.5. Therefore all data could be used for analysis and no transformation of the data was required.

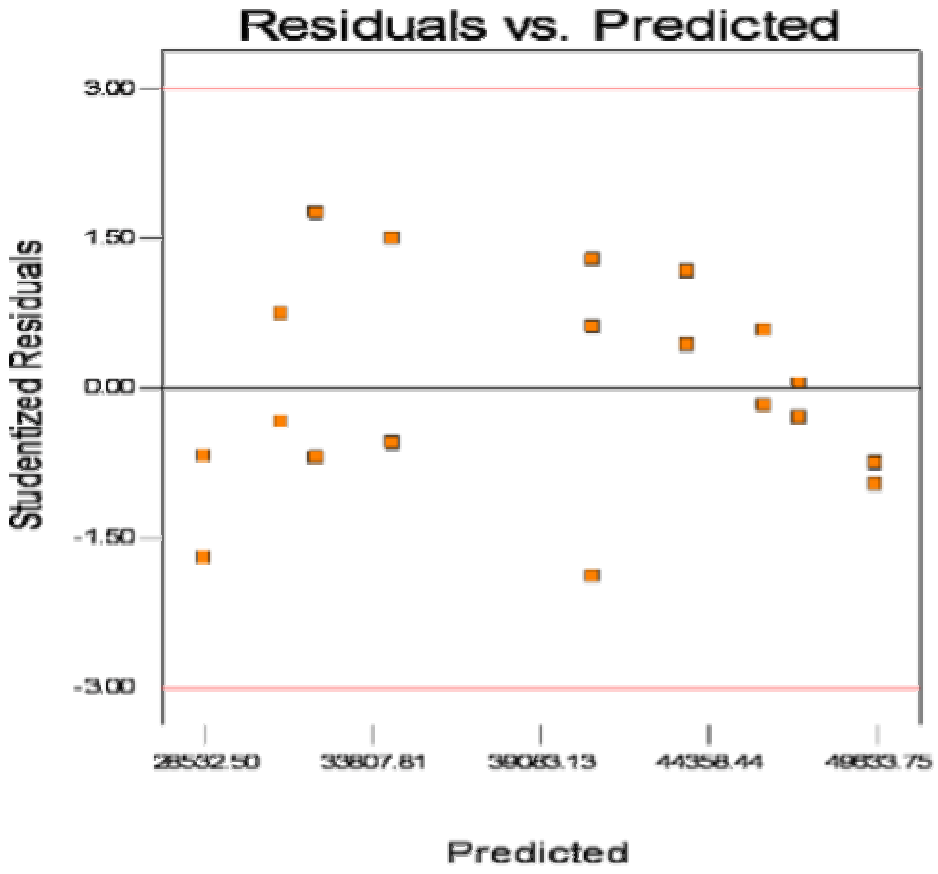

Figure 20 The residuals vs. predicted plot that means that the studentized residuals (predicted – actual values) against the predicted values are plotted together

The correlation coefficient for the predicted vs. actual plot is 0.958 (Figure 21), showing the model to accurately represent the collected data. No curvature was found in the design; therefore the system can be viewed as linear, and no further experimentation (such as a response surface experimental design) was necessary.

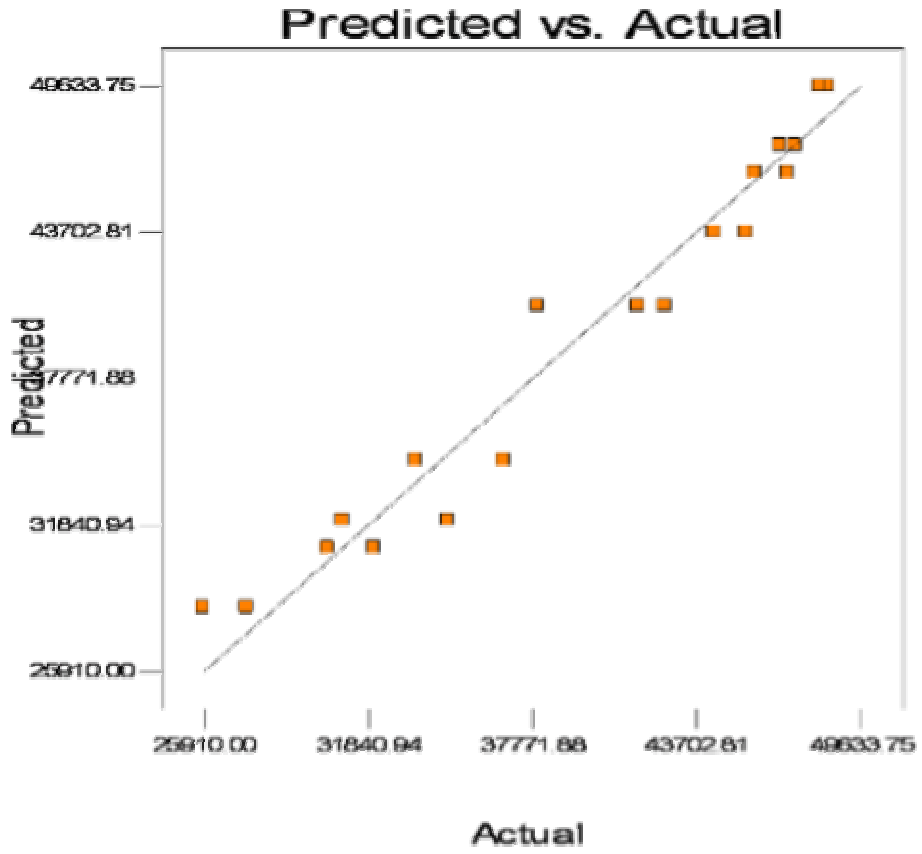

Figure 21 The predicted vs. the actual values should give a linear regression

5.5.2 Peptide analysis by SDS-PAGE

Each digested sample of the factorial design was loaded on a 15% SDS-PAGE to see the corresponding molecular weights of the hydrolysates produced. If the samples with high level of pancreatin concentration (Run 1-6; 14; 19) are viewed together, the pattern of the bands (big smear around 10kDa) are all the same. The samples with a low level of pancreatin concentration (Runs 7; 8; 12; 13; 15-18) show all approximately the same high molecular weight bands in addition to the low molecular weight bands. The centre point (Run 9) is between both; it shows the low molecular weight bands but fewer high molecular weight bands than the samples with a low level of pancreatin concentration. It seems again that the enzyme concentration of pancreatin is the most influencing factor.

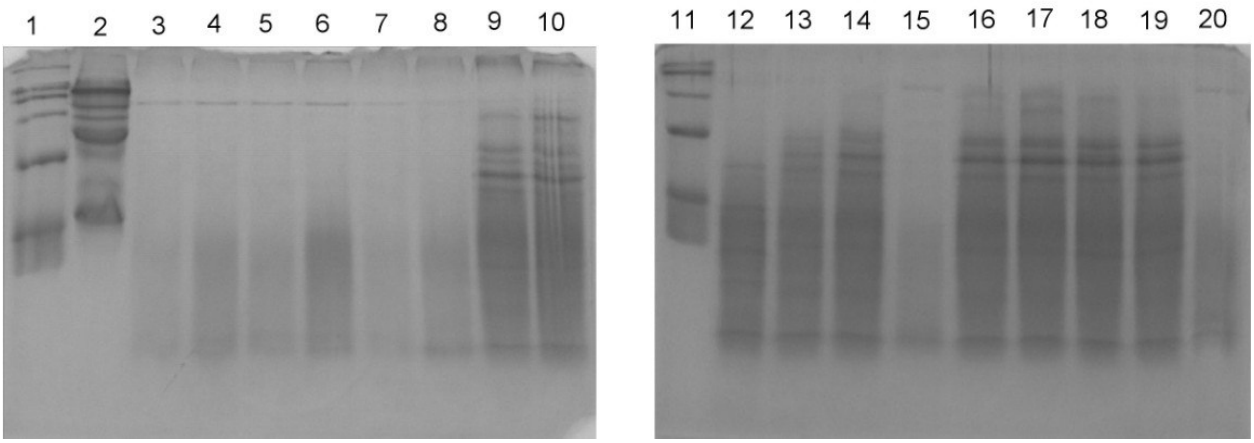

Figure 22 The digested samples of the factorial design loaded on a 15% SDS-PAGE (coomassie blue staining) to investigate their polypeptide profile.

Well **1:** Broad range Marker; **2:** SPI undigested; **3:** Run 1; **4:** Run 2; **5:** Run 3; **6:** Run 4; **7:** Run 5; **8:** Run 6; **9:** Run 7; **10:** Run 8; **11:** Broad range Marker; **12:** Run 9; **13:** Run 12; **14:** Run 13; **15:** Run 14; **16:** Run 15; **17:** Run 16; **18:** Run 17; **19:** Run 18; **20:** Run 19

To obtain a better overview of the polypeptide profile analyzed by SDS-PAGE, specific bands of each sample were compared for their presence or absence. Using this data UPGMA (unweighted pair-group method using arithmetic averages) clustering analysis method based on percentage disagreement was created. According to Figure 23, the samples with a high level of pancreatin concentration form a single large group, where the samples with a low level of pancreatin concentration build different small groups. All digested samples are shown to be quite dissimilar to the undigested soy protein isolate.

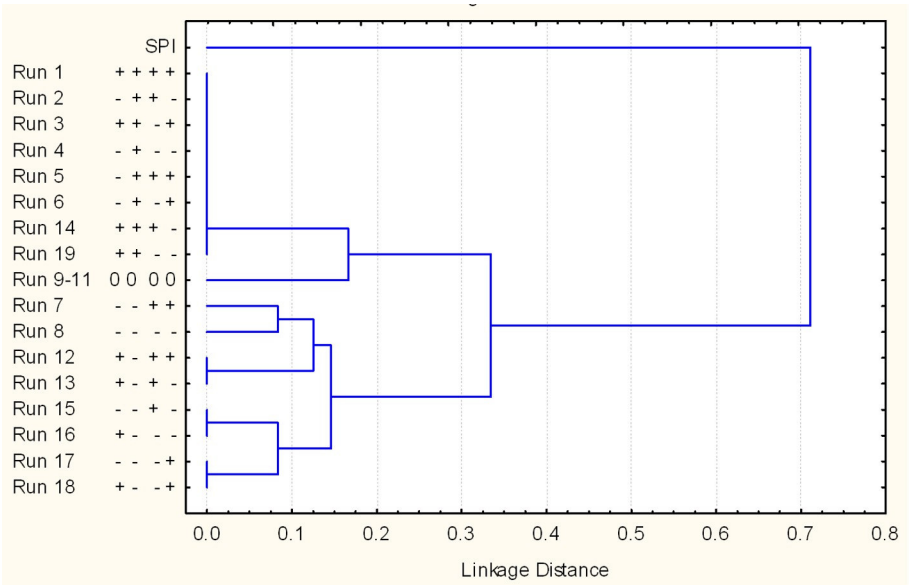

Figure 23 Dendogram: UPGMA clustering analysis for SDS-PAGE results based on percent disagreement from the presence/absence of bands for the 18 runs

5.5.3 Filtration

To see the influence of the peptide concentration on the filtration step, 3 different samples (+ + + +; - - - -; centre point) of the factorial design were sequentially filtrated with the 3kDa and 1kDa membranes by dead-end filtration.

As expected, the production of more peptides (smaller molecules) leads to a faster filtration (with the + + + + sample). However with a maximum flux of 1.5 L/(m^2*h) the filtration is considered extremely slow. The centre point had a flux of 1.3 L/(m^2*h) and the - - - - sample a flux of 0.9 L/(m^2*h).

The filtration of the + + + + sample with the 1kDa membrane after the 3kDa membrane was unexpectedly fast, compared to the other two samples.

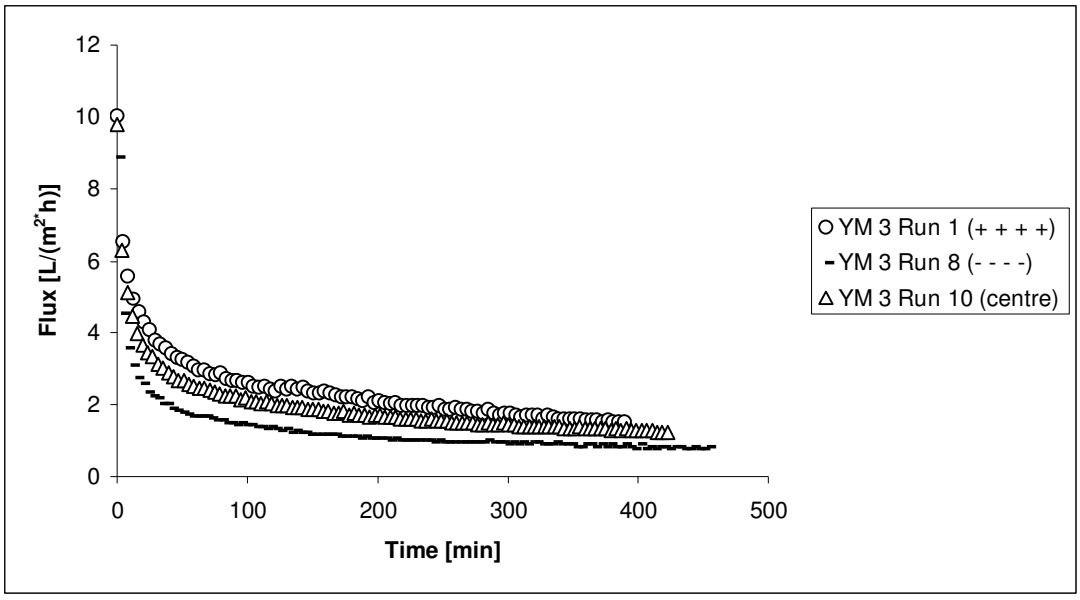

Figure 24 The permeate flux of the dead-end filtration of the + + + + ; - - - - and the centre point of the factorial design with the 3kDa membrane as a function of time

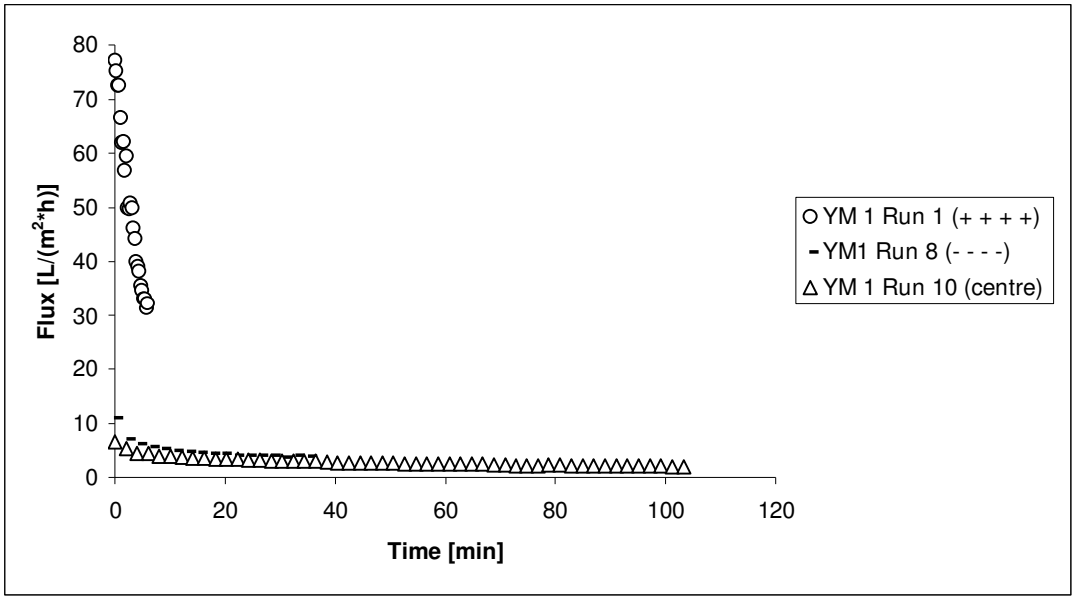

Figure 25 The permeate flux of the dead-end filtration of the + + + + ; - - - - and the centre point of the factorial design with the 1kDa membrane as a function of time

A mass balance of the 3kDa and the 1kDa filtration of every sample was established Through two different methods, by peptide concentration (as equivalent Phe-Gly) and total solids. In general, both methods showed approximately the same results. The 3kDa filtration had almost no loss in comparison to the 1kDa filtration. However, for all runs on the 3kDa membranes a significant cake was formed, this was about 4-6% of the total solids. In the permeate of the 3 kDa filtration between 11 and 24% of the total peptides could be found, depending on the run. In the 1kDa membrane filtration, the peptide contents in the permeate are slightly higher, between 16 and 27%, depending on the digestion conditions. In general from the run 1 (highest peptide concentration) the most peptides could be recovered.

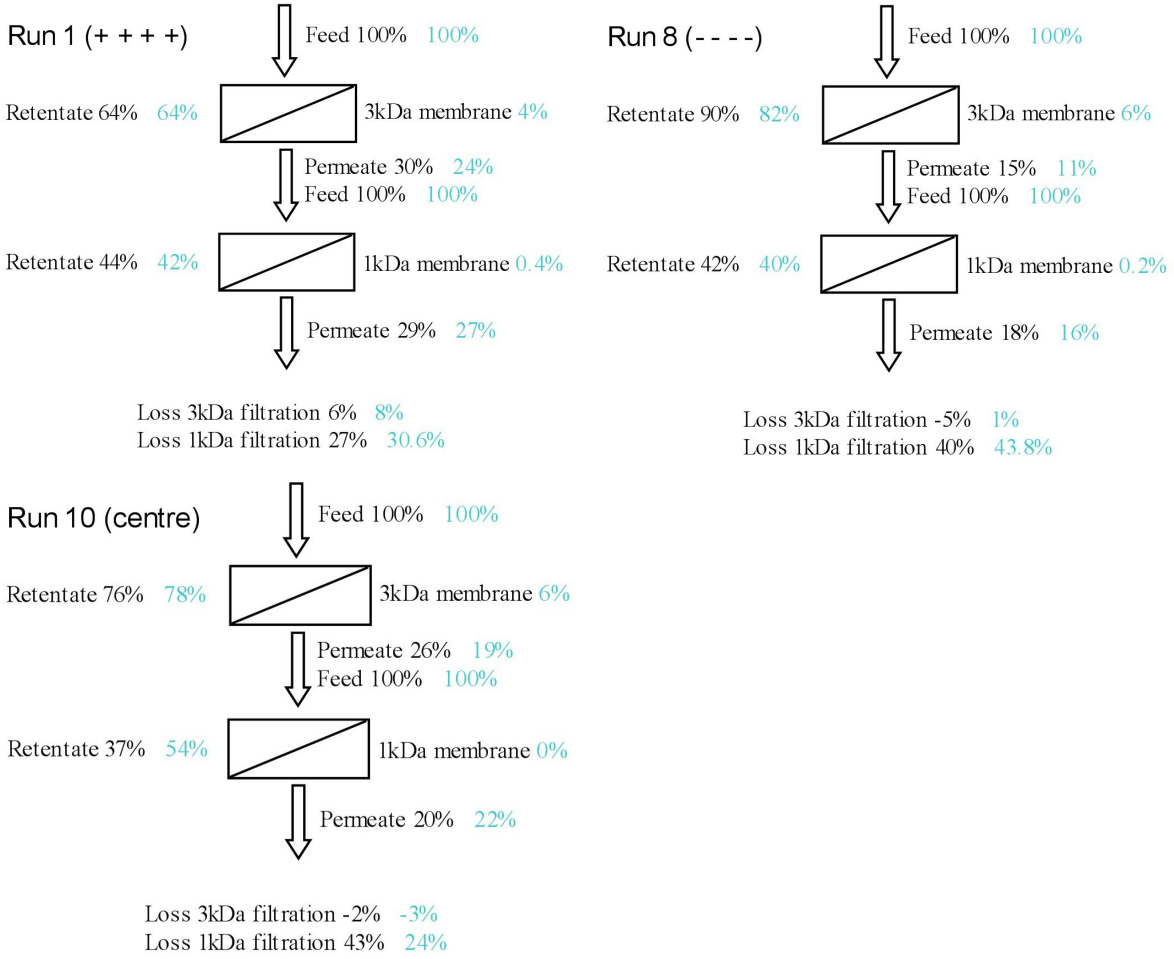

Figure 26 Massbalance of the 3kDa and the 1kDa filtration of the three different digestion samples **(+ + + +;** centre point; - - - -) with the peptide concentration in black and the total solids in **turquoise**

5.5.4 Antioxidant analysis by DPPH assay

After freeze drying of the permeate and retentate of the three filtrated samples (+ + + +; centre point; - - - -) they were analyzed by the DPPH assay to investigate the antioxidative properties.

As shown in the literature the antioxidative peptides should have a molecular weight between 700 and 2500 Da, consisting by 3 to 16 amino acid residues. [3] Because the calibration curve was prepared with ascorbic acid (0 / 0.2 / 0.3 / 0.4 / 0.5mM), the results of the DPPH assay are shown in mg equivalent ascorbic acid / g peptide. According to Figure 27 the antioxidative properties are the highest in the retentate of the 1kDa filtration. Highest estimated antioxidative properties (1.6 mg equivalent ascorbic acid / g peptides) were found for run 1 (+ + + +). The permeate of the 3kDa membrane was estimated at 0.3 mg equivalent ascorbic acid / g peptides, and the permeate of the 1kDa membrane was estimated as 0.9 mg

equivalent ascorbic acid / g peptide. The antioxidative properties of the run 8 (- - - -) were highest in the retentate of the 1kDa filtration with an amount of 1.5 mg equivalent ascorbic acid / g peptide. In the permeate of the 3kDa filtration a concentration of 0.4 mg equivalent ascorbic acid / g peptide was estimated and a concentration of 0.7 mg equivalent ascorbic acid / g peptide was measured for the permeate of the 1kDa filtration. According to these results it looks like the antioxidative peptides have a smaller size than 3kDa but a bigger size than 1kDa. It also seems like a higher amount of peptides yield in a higher amount of antioxidative properties.

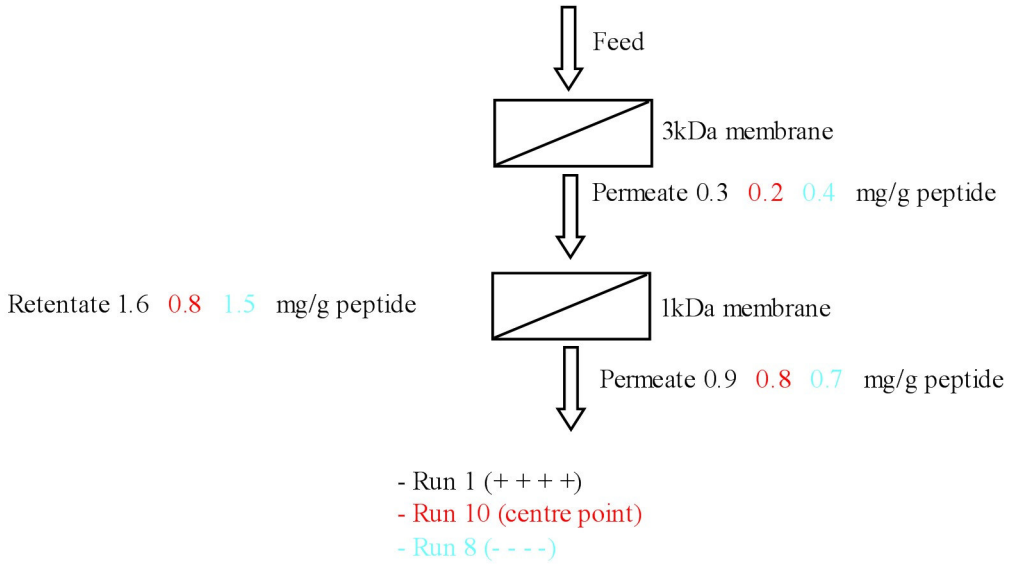

Figure 27 Antioxidative properties in mg equivalent ascorbic acid / mg peptide of all three digestion samples (+ + + +; - - - -; centre point) for each filtration step (n=3)

5.5.5 Summary for filtration and antioxidant properties

A summary of the data from the filtrations of the 3 digestion samples of the factorial design is presented in Tables 13 - 15. All tables show the total solids, the peptide concentration (as equivalent Phe-Gly) and the antioxidative properties. For each filtration the feed, retentate and the permeate were analyzed.

It is conspicuous that for each of the 3 digestion samples, the total equivalent ascorbic acid in the retentate of the 1kDa is higher than the Feed of the 1kDa filtration. The production of more antioxidative peptides is not realistic. For the determination of antioxidative properties using the DPPH assay, only one chemical reaction under specific conditions is used. Therefore the presentation in total antioxidative activity is inappropriate and can't be generalized. [13]

The permeate of the 1kDa filtration shows the highest g of peptides / g of solids.

Table 13 Total solids, peptide and antioxidant analysis of the filtration of digestion sample run 1 (+ + + +)

	Feed 3kDa	Retentate	Permeate	Feed 1kDa	Retentate	Permeate
total solids [g]	4.7	3.0	1.1	1.1	0.5	0.3
total solids [%]	100.0	63.8	23.5	23.5	42.1	26.8
peptides [g]	1.09	0.70	0.32	0.32	0.14	0.09
peptides [%]	100.0	64.0	29.5	29.5	43.8	29.0
peptide/solids [g/g]	0.2	0.2	0.3	0.3	0.3	0.3
total equiv. Asc. Acid [mg/L]	-	-	100.9	100.9	218.4	88.0
equiv. Asc. Acid [%]	-	-	-	100.0	216.3	87.2
equiv. Asc. Acid/g solids	-	-	342.0	342.0	527.1	328.8
equiv. Asc. Acid /g peptide	-	-	0.3	0.3	1.5	0.9

Table 14 Total solids, peptide and antioxidant analysis of the filtration of digestion sample run 9 (centre point)

	Feed 3kDa	Retentate	Permeate	Feed 1kDa	Retentate	Permeate
total solids [g]	4.0	3.1	0.8	0.8	0.4	0.2
total solids [%]	100.0	78.2	19.0	19.0	54.2	22.1
peptides [g]	0.91	0.69	0.24	0.24	0.09	0.05
peptides [%]	100.0	75.9	25.8	25.8	36.7	19.9
peptide/solids [g/g]	0.2	0.2	0.3	0.3	0.2	0.3
total equiv. Asc. Acid [mg/L]	-	-	58.1	58.1	67.6	37.6
equiv. Asc. Acid [%]	-	-	-	100.0	116.4	64.7
equiv. Asc. Acid/g solids	-	-	257.7	257.7	194.0	241.9
equiv. Asc. Acid /g peptide	-	-	0.2	0.2	0.8	0.8

Table 15 Total solids, peptide and antioxidant analysis of the filtration of the digestion sample run 8 (- - - -)

	Feed 3kDa	Retentate	Permeate	Feed 1kDa	Retentate	Permeate
total solids [g]	4.4	3.6	0.5	0.5	0.2	0.1
total solids [%]	100.0	82.1	11.5	11.5	40.4	15.7
peptides [g]	0.62	0.56	0.10	0.10	0.04	0.02
peptides [%]	100.0	90.5	15.5	15.5	41.6	17.5
peptide/solids [g/g]	0.1	0.2	0.2	0.2	0.2	0.2
total equiv. Asc. Acid [mg/L]	-	-	39.2	39.2	60.7	11.1
equiv. Asc. Acid [%]	-	-	-	100.0	155.1	28.3
equiv. Asc. Acid/g solids	-	-	355.4	355.4	334.2	184.9
equiv. Asc. Acid /g peptide	-	-	0.4	0.4	1.5	0.7

6 Discussion

6.1 Digestion of soy protein isolate from ADM

6.1.1 Solubility of SPI

The solubility of the soy protein isolate (SPI) has a large influence on digestion performance. With increasing solubility, greater reaction rates are seen due to higher substrate accessibility for the digestive enzymes. During the digestion protocol two different pH levels are used, a pH of 1.5 for the initial pepsin digestion and a pH of 7.8 for the subsequent pancreatin digestion. In each digestion similar solubilities are desirable. As shown in (Figure 4) solubility around the isoelectric point (4.6) is zero. If pH is deviated from the isoelectric point in either a positive or negative direction, a corresponding increase in solubility is attained. Solubility of the SPI is greatest at a pH of 11. At the pH levels of 1.5 and 7.8, the solubility is approximately 38% in both cases, giving similar SPI solubility during both digestion steps.

The soy protein isolate consists of two major globulins, the β-conglycinin and the glycinin whose quaternary structures are dependent on the pH and the ionic strength. Above the pI of the soy protein (4.6) the charge of the globulins is positive. If the pH is lower than the pI, the charge is then negative. [5]

According to the literature the soy protein isolate shows a high solubility of about 90% at alkaline conditions when measured by the absorbance at 280nm. [25] In the established solubility experiment (Figure 4) it was shown that at an alkaline pH of 11 the solubility is the greatest, however the SPI solubility via Bradford method is estimated as 47%. Therefore it is possible that the established Bradford method used during the course of this work underestimates protein content. The measurement of total soy protein content in solution by Bradford was chosen due to reagent and instrument availability. The Bradford method preferentially binds aromatic amino acids and arginine. [26] Therefore if a protein contains a smaller number of aromatic amino acids and arginine, protein concentrations will be underestimated. Another method as Kjeldahl, Lowry or Buriett would be a choice to consider. [27]

6.1.2 Comparison of different digestion enzymes

Absorbance measurements taken by spectrometer for the OPA assay, to determine peptide concentrations, during a digestion contained small amounts of measurement error. Therefore all samples were measured in quadruplicates to receive a representative mean. Due to this measurement error triplicates of each digestion method (pepsin only / pancreatin only / pepsin & pancreatin) were necessary to show a good reproducibility (Figure 5). Triplicate digestions yielded variations of less than 10% allowing different digestion protocols to be compared.

The blend of enzymes in the pancreatin leads to a greater number of cleavage positions which should, in comparison to pepsin, yield a greater variety of peptides after digestion. Pancreatin contains endolytic and exolytic proteases; therefore the hydrolysis will occur within the polypeptide chain and at the end of a polypeptide chain. The pepsin is an endolytic enzyme and will therefore cleave within the polypeptide chain. The action of the pancreatin is higher if a pre-treatment with pepsin is first performed (Figure 5). When used on their own, a digestion with either pepsin (30 min digestion) or pancreatin (60 minute digestion) leads to a similar peptide content determined by the OPA method. When the two enzymes are used in sequence a greater peptide yield is observed (Figure 5). From these results it is hypothesized that pepsin cleaves more effectively cleaves larger peptides, where as pancreatin cleaves more effectively small peptide chains.

Antioxidative soy peptides are composed of 3 to 16 amino acid residues. [3] It is expected that a high release of peptides during a digestion would yield a higher antioxidative activity. Therefore it is expected that a digestion utilizing both pepsin and pancreatin would yield hydrolysates with higher antioxidative activity.

A soy protein isolate control digestion (without any addition of enzymes) was completed to see the influence of the increased temperature on the hydrolysis. Another method of protein hydrolysis is through heat treatment. However as shown in Figure 5, a temperature greater than those used in these experiments (37°C and 40°C) would be needed. During each control experiment the peptide concentration was stable. Variation was less than 10% as also shown for the digestion experiments.

6.1.3 Influence of pH and SPI concentration on Pepsin digestion performance

The digestion with pepsin produced the same amount of peptide for a pH of 1.5 / 2.0 or 2.5. If the ratio of enzyme to protein is taken into account, the initial concentration of SPI (5% w/w or 3.12% w/w) did not affect the final yield (peptides produced/original SPI concentration) of peptides (Figure 6). Therefore the enzyme is not the limiting factor in the reaction; it is more likely the solubility of the soy protein isolate that limits the reaction.

6.2 Digestion of soy protein isolate from Solae

6.2.1 Comparison of Solae and ADM SPI

Industrially produced soy protein isolates can be very different in their physicochemical properties due to their processing conditions (extraction, purification and drying) of different suppliers. These differences could cause different behaviour in further food production and final food properties such as consistency and taste. [28]

The soy protein isolate from ADM and Solae showed different behaviours during TGA analysis (Figures 12 and 13). Therefore the SPI from ADM and Solae could be expected to be slightly different in their physicochemical properties.

6.2.2 Comparison of Solae and ADM SPI digestions

The SPI substrates from ADM and Solae yielded different digestion results. The Soale SPI substrate contained initially a greater amount of peptides. According to figure 7 the yield of peptides after the two step digestion (30min at 37°C and pH 1.5 with pepsin and 60min at 40°C and pH 7.8 with pancreatin) was higher with the Solae substrate. During the pepsin digestion no significant difference in peptide generation was seen for the two different SPI. However, it was observed that the peptide generation during the pancreatin digestion was greater for the Solae SPI. Therefore, it can be said that due to the initial difference in globulin properties and the differing degree of initial hydrolysis between the two substrates, a greater peptide yield was found for the Solae SPI. The differing degrees of initial hydrolysis and globulin concentrations for the two different SPI is most likely due to the different soybeans employed by ADM and Solae.

6.2.3 Filtration of Solae and ADM SPI Hydrolysates

A filtration with a 3 kDa membrane resulted in approximately the same flux for both hydrolysates. In the filtration with the 1 kDa membrane (Figure 8) the ADM hydrolysate led to a higher flux than did the Solae hydrolysate. Because the filtrations were carried out by stirring the fouling mechanisms can not be studied. To evaluate membrane fouling mechanisms non-stirred filtrations would need to be performed. A more effective method of filtration is cross flow filtration. Cross flow filtration reduces cake formation and a higher flux could be expected but requires a larger volume of feed solution.

6.2.4 Mass balance during a filtration

A mass balance was performed with the sample of the SPI from Solae to see the peptide fractionation during the two filtration steps. For the 3kDa filtration, the concentration of peptides in the retentate and the permeate was measured. The retentate and permeate of the 1kDa filtration was measured for the concentration of peptides and the total solids. The total solids for the 3kDa filtration could not be measured because the samples were in the freeze dryer when the experiment for the 1kDa filtration was carried out.

After the 1kDa filtration 36% of the total solids content was found in the retentate and 27% in the permeate. According to the peptide concentration 107% of the peptides of the feed were in the retentate and 99% were in the permeate. A production of peptides during the filtration is not realistic. Therefore the results attained for the total solids should be considered to be representative but not the peptide analysis..

For the 3kDa filtration, approximately half of the peptides in the feed (digestion solution) were recovered in the retentate and only 15% were recovered in the permeate. This balance is more realistic for the peptide concentration, although a loss of 35% is significant.

The calculated mass balances were not representative of the physical separation process. The hold up volume of the filtration unit should be ~1.2ml, therefore the calculated loss is larger than realistically possible. The building of a cake on the membrane during the filtration could account for part of the losses, especially for the 3kDa filtration, however a loss of around 35% on the membrane is too large and again not realistic. Another problem could be the work with small volumes. A larger amount of volume reduces the loss.

A full mass balance for a 3kDa and a 1kDa filtration should be established through the measurement of the total solids and the peptide concentrations. Through the evaluation of this type of data a relationship between peptides and totals solids could be defined.

6.3 Filtration of SPI hydrolysates from ADM

6.3.1 Maintenance of the membranes

The functionality of the membranes was evaluated via a water flux prior to each experimentation. This was important to ensure that the membranes had similar functional properties before starting each filtration run. In the case where membranes are reused this aspect is even more important, as improper washing after a filtration can leave particles in the membrane structure having a significant influence on the next filtration. The membranes in that case weren't reused. A new membrane was always taken. The used membranes were stored in 10% ethanol for possible reuse and/or analysis. Future cleaning should occur directly after the filtration run as it was discovered that after some weeks fungus colonized the membrane surfaces. The membrane could be washed with either alkaline solutions, cleaning solution (as Terg-A-zyme) or, for extremely dirty membranes, proteases. [20]

As discussed, the water fluxes (see appendix part 11.5) of the 1kDa and 3kDa membranes were always evaluated before each experiment. Calculated standard deviations (n = 5) for the water flux of the 3kDa and the 1kDa membranes were 9 and 15% respectively. Water flux curves had associated correlation coefficients greater than 0.99 for all experimental runs. The water flux variation is probably due to slight differences in the manufactured products. The measured fluxes are in the range of the expected values from Millipore (the supplier of the membrane). The measured water flux of the 1kDa membrane at 50 psi was around 19 L/(m^2*h) while Millipore suggest a water flux between 12-24 L/(m^2*h) at 55 psi. The obtained water flux at 50 psi for the 3kDa membrane was around 40 L/(m^2*h) while Millipore suggests a water flux of 36-48 L/(m^2*h). Therefore the measured water fluxes are in the suggested range, and were considered suitable for experimentation. Membrane functionality is not expected to be reduced when running low fluxes. The filtration with the YM membranes used is for diluted solutions. Therefore a very slow flux of the soy protein digestion can be expected. An alternative membrane material (polyethersulfone) is often used for higher concentrations, and gives good recovery and a high flux. This membrane material would most likely be a better choice and should be considered as a next step in the future experiments. [20]

6.3.2 Comparison of different digestion methods for SPI from ADM

A 3kDa filtration followed by a 1kDa filtration (Figures 10 and 11) was performed for 3 different digestion methods (pepsin-only, pancreatin-only, pepsin and pancreatin). The filtration flux for the 1kDa membrane was approximately the same for the 3 investigated digestions. The 3kDa filtration with the pancreatin-only digestion resulted in a higher flux than the 3kDa filtration with the pepsin only digestion. As previously discussed the pancreatin-only digestion produced larger peptides and the pepsin-only digestion produced smaller peptides. It is suggested that the smaller peptides created with the pepsin-only digestion contributed to the observed increase in membrane fouling.

The flux for the 3kDa filtration in non-stir mode was 2 L/(m^2*h). The flux for the 3kDa filtration when utilizing stirring was to 7 L/(m^2*h). Neither flux value is very high. For the 1kDa filtration, a flux of around 13 L/(m^2*h) could be reached when utilizing stirring.

An alternative membrane material (polyethersulfone) is often used for higher concentrations, and gives good recovery and a high flux. The utilized regenerated cellulose material is appropriate for diluted solution and therefore extreme fouling can be expected. Use of polyethersulfone would decrease membrane fouling and increase filtration flux. Another suggested method to increase flux would be to use a cross flow filtration system. In this case a higher flux would also be expected because cake formation would be limited but a cross flow system requires larger volumes.

6.4 Characterisation of the soy protein isolate and its peptides

6.4.1 TGA profile of SPI

Different ash content of the two different SPI samples (ADM and Solae) were obtained via TGA. The ash content of the ADM sample was much larger than for the Solae sample. The ADM SPI showed only one peak which contained a large amount of background noise. The Solae SPI showed two peaks. Because the soy protein consists mainly of the two globulins, glycinin and β-conglycinin, the two peaks could be seen as the two globulins. Because the β-conglycinin denatures at a lower temperature than the glycinin, the first peak in this analysis could be considered as the β-conglycinin. The difference in the TGA results for the 2 different SPI could be due to a slightly different composition or a different production method. [28] [28] It is expected that as the two SPI were slightly different one would expect to see differences in the digestion and filtration performances.

6.4.2 Peptide analysis by MALDI-TOF

To compare the peptides produced for the three different digestions (pepsin-only / pancreatin-only / pepsin & pancreatin), MALDI-TOF was used. Already in the undigested soy protein isolate, some peptides with a mass between 500 and 1500 Da are present. If the SPI is digested with pepsin only, several more peptides with a molecular weight between 500 and 4000 Da are detected. The digestion with pancreatin only led to a lower peptide production in the investigated mass range. Only peptides with a molecular weight between 500 and 2000 Da were detected with the pancreatin only digestion. The largest number of peptides was produced with the two step digestion. A large number of different peptides between 500 and 4000 Da were produced. Several of the peptides produced were quite numerous. As expected, it seemed that the pancreatin acts more effectively if presented with pepsin cleaved globulins. The pancreatin can be considered to more effectively cleave smaller peptide chains.

The MALDI-TOF can detect masses up to 100kDa. Therefore the β-conglycinin and glycinin couldn't be detected with this method. It would be interesting to see on which subunits the two different enzymes act. To monitor the different subunits of the two globulins in the SPI a pre-treatment with β-mercaptoethanol could be performed, as this reagent cleaves the disulfide bonds between the subunits. The different subunits would then have molecular weights smaller than 100kDa and could be detected by MALDI-TOF. [28]

MALDI-TOF was a good choice for the analysis of the different digestions. The unpurified digestion samples contained a number of different peptide sizes and other impurities such as enzymes and salt. However MALDI-TOF was able to give a good resolution in all cases. Other methods to determine different masses of samples such as ESI (electron spray ionisation) would not be capable of analyzing such impure samples.

6.4.3 Peptide analysis by SDS-PAGE

The SDS-PAGE was used for characterising the two enzymes pepsin and pancreatin, to investigate the different subunits of glycinin and β-conglycinin and to compare the different peptides produced via the three different digestion methods (pepsin-only, pancreatin-only, pepsin & pancreatin). The molecular weight of each band could be calculated according to the marker employed.

The theoretical sizes of the subunits of glycinin are between 37 and 40 kDa for the acidic polypeptide chain (A) and between 19.9 and 20 kDa for the basic polypeptide chain (B). The β-conglycinin has theoretical sizes for the subunit α' between 57 and 83 kDa; for the subunit α between 57 and 76 kDa and for the subunit β between 42 and 53 kDa. [5] The estimated molecular weights of the different subunits of β-conglycinin were in the range of the theoretical values, but the ones for the glycinin were slightly out of the range by about 5 kDa for the chain B and about 2 kDa for chain A. This small amount of error is not too large and is expected as the theoretical values are already given in a range. As well, different studies have reported a range of molecular weight values.

The molecular weight and purity of the enzymes pepsin and pancreatin were determined by SDS-PAGE. For the pancreatin, different bands were expected and observed, as the pancreatin is a blend of different enzymes. However pepsin should give only 1 band. For this enzyme different bands were obtained as well. The molecular weight of pepsin is 34.6kDa and after the β-mercaptoethanol treatment four different smaller subunits should be obtained. The higher molecular weight band at vales of 80kDa may be indicative of possible impurities.

The analysis of the different digestion samples (pepsin only / pancreatin only / pepsin & pancreatin) via SDS-PAGE showed different peptide size distributions. The digestion with pepsin only or pancreatin only, showed a large number of high molecular weight polypeptides. The high molecular weight bands remaining could be related to the globulins glycinin and β-conglycinin. Considering that, it seemed that the pepsin digested more effectively the globulin β-conglycinin than does the pancreatin. The digestion with pepsin & pancreatin showed a smear around 10kDa. Therefore a large number of peptides were created around the 10 kDa range and it can be said that a better hydrolysis could be achieved.

The samples analyzed after the filtration did not show any bands. It can be assumed that the concentrations within these samples are too low. Use of a silver staining solution, instead of the commassie blue solution, could be used to obtain a higher sensitivity. Considering the molecular weights of the peptides in the 3kDa permeate and in the 1kDa permeate, it could also be that the peptides were running out of the gel. But usually, as used in the performed SDS-PAGE the commassie blue molecule in the sample buffer has an approximate molecular weight of 500Da and produces a front which can be visibly seen on the gel. Therefore all molecules with a size greater than 500Da should be still in the gel. Therefore the SDS-PAGE method should show the peptides of interest. Antioxidative peptides are said to have a size

range between 700 and 2500Da and therefore will not run past the commassie blue front and off the gel. [3]

6.5 2^4 Factorial design to investigate digestion parameters

6.5.1 Digestions

A 2^4 factorial design was used investigate the effect of digestion parameters on the overall digestion performance (based on final peptide concentration). A factorial design was chosen because it is easier to interpret the results, fewer experiments for the same information are necessary and a model can be created. With the execution of the 3 centre points the variation could be evaluated. If the variation is too large the factorial design does not yield useful information pertaining to the factors studied. In this case a good variation of 4.5% between the 3 centre points could be obtained.

The concentration of the enzymes and the respective incubation times were chosen as the most important factors affecting the production of peptides in the digestions and were therefore studied using a factorial design. Digestion time can have an effect on final peptide concentration due to the varying contact time between the substrate and the enzyme. The concentration of enzyme can influence reaction times, however if the substrate is the limiting reactant, an increasing amount of enzyme does not help to produce more peptides.

Through the factorial design a general linear model could be generated to investigate the peptide concentration. The correlation coefficient of the regression has a value of 0.958 and is therefore very good. Therefore the model is linear and no curvature exists. That was the reason no further experiments were performed. A full response surface was therefore not necessary.

A transformation of the data is sometimes necessary to receive a better model fit. In the case here no transformation was necessary according to Figure 20 in part 4.5.1.

Through the establishment of the linear equation, representing the peptide yield, the influence of each factor could be investigated. The higher the coefficient for a given factor, the more important is the influence of the corresponding in the model. The execution of the factorial design showed that the concentration of the pancreatin is the most important factor of the four studied (coefficient of 7.59). The second most import factor was the time of pancreatin during

digestion (coefficient of 1.76). The third influencing factor is the time of pepsin during the digestion (coefficient of 1.21). It was also found that the concentration of pepsin and all interaction terms did not significantly influence the production of peptides. Therefore the low level of pepsin concentration could be used for future digestions. To obtain the highest peptide concentration, the theoretical digestion parameters should be taken as followed: a final pancreatin concentration of 2.5g/L, a final pepsin concentration of 0.15g/L, an incubation time for pepsin for 45 minutes and 120 minutes for pancreatin.

6.5.2 Peptide analysis by SDS-PAGE

All hydrolysate samples from the factorial design were loaded on an SDS-PAGE to determine the molecular weights of the different polypeptides. It was seen that all digestion with a high concentration of pancreatin had a similar pattern, with a broad smear around 10kDa. For the samples with a low concentration of pancreatin, different patterns were obtained. In the low pancreatin concentration samples, high molecular weight bands related to the different subunits of conglycinin and β-conglycinin were still visible.

The smaller peptide molecules could not be resolved very well via SDS-PAGE. With the method of Laemmli, only molecules higher than a size of 20kDa were separated and well resolved. The smaller peptides may not or be only partially separated from the bulk of SDS and therefore stay in the stacking gel. This could be verified easily by staining the stacking gel as well. A different pH or a different concentration of the acrylamide of the stacking gel could also help the peptides behave differently. With the addition of urea in the separating gel, a different behaviour of the smaller molecules could be achieved. Methods specially developed for peptides use, for example, another buffer system such as MES-buffer with 6M urea. The use of tricine instead of glycine is sometimes used or the use of electrolytes in a one layer gel (without stacking gel) is also sometimes performed. A method utilizing no stacking gel and added electrolytes was used to analyse the different digestion samples from the factorial design, but was not successful. If the method is performed as described in the literature [30] the bands appear as a smear. Only 4 of the 6 bands of the marker could be resolved. Further work would need to be completed to bring this method to an effective level. [21] [22]

Based on the SDS-PAGE analysis, the different bands were evaluated according to their absence or presence, and a matrix was established. Based on that matrix, a percentage of dissimilarity between the runs could be calculated and visualized in a clustering tree. A single group containing all runs with a high concentration of pancreatin was formed. The other 8 runs were separated into different subgroups. The two main conclusions from these results are that all samples are very different from the original undigested SPI, and that a high concentration of pancreatin leads to a significantly different peptide pattern.

6.5.3 Filtration

The 3kDa filtration showed that a lower peptide concentration led to a lower flux. Therefore smaller peptides led to a higher flux. It could then be assumed that larger peptides block the membrane and therefore decrease the flux. The 1kDa filtration did not follow a similar trend. The sample with the higher concentration of peptides led to an unexpected fast flow, while the other two samples were as slow as the 3kDa filtration. The centre point of the 1kDa filtration had the slowest flux, therefore it can't be concluded that a lower concentration of peptides leads to a slower flux.

The peptide and total solids mass balances (Figure 26) for the three filtrated samples (+ + + +; - - - -; centre point), gave approximately the same results (in percentage); therefore the total solids can be said to be directly related to the peptide concentration. A recovery between 11 and 24% of the peptides in the 3kDa membrane and between 16 and 27% in the 1kDa membrane is not very high. The assumption is that the other peptides are either too big or stuck in the cake. On the 3kDa membrane a mass of about 5% of the total solids were estimated in all 3 samples.

6.5.4 Antioxidant analysis by DPPH assay

Antioxidant analysis using the DPPH assay are shown in mg equivalent ascorbic acid / g peptide, as the calibration curve was prepared with ascorbic acid. Therefore these results cannot be related to real antioxidative units, but can be used for the comparison of the samples in this work.

In executing the DPPH assay, the absorbance of the DPPH solution decreases over time. Therefore a ratio of the final absorbance divided by the initial absorbance was used to establish the standard curve and to calculate the samples according the standard curve. Sample absorbance could be affected partly by light influence or by the changing amount of

oxygen amount in the water or air. Because the solubility of oxygen in water is highly dependent on the temperature, the change of temperature could also affect the drop in absorbance. To be sure that oxygen does not influence the DPPH assay, the solution could be sparged with N_2 to remove oxygen from the solution, and the assay should be carried out at a consistent temperature. The bottle used for the solution was covered with aluminium foil so that a minimum amount of light could penetrate the bottle and interfere with the radicals. [31]

The samples right after the digestion and the retentate of the 3kDa membrane couldn't be analysed because of precipitation after mixing the components during the execution of the assay. As known from the literature, protein precipitation could occur because the medium of the assay contains an aliphatic alcohol. Therefore another assay for the antioxidative properties should be considered to carry out, because of the interference with the non filtrated samples. There might be a problem of hydrophilic and hydrophobic compounds in the samples which would also interfere with the alcohol in the medium. [32]

According to Figure 27, the sample with the highest concentration of peptides in the feed of the 3kDa filtration (sample + + + +) yielded in the greatest amount of antioxidative properties in the permeate of the 1kDa filtration. The lower the concentration in the feed of the 3kDa filtration, the lower the antioxidative properties. Therefore it can be concluded that a high concentration of peptides leads to high antioxidative activity in the 1kDa permeate. The production of even a higher concentration of peptides would probably lead to a higher antioxidative activity.

The estimated values did not yield in a very high number of antioxidant activity. But it could be that there is a problem with the assay. The undigested, unfiltered SPI couldn't be analyzed by DPPH, because of precipitation problems. If a greater amount of antioxidative activity is obtained during the digestion could not be proved but could be suggested. According to the supplier, the used ADM SPI should contain a low concentration of isoflavones and therefore a higher antioxidative activity in comparison to other commercially available SPI should be present.

7 Conclusions

The OPA assay was shown to be a good method for the measurement of the production of peptides during a digestion. The reproducibility of the OPA assay fell within acceptable limits.

The execution of the DPPH assay for the measurement of the antioxidative properties worked well but was not as robust as would have been preferred. Only the samples after the filtration could be evaluated for their antioxidative activity. Samples after digestion or the retentate of the 3kDa membrane could not be analysed due to precipitation.

The factorial design study showed the pancreatin concentration to have a dominant influence on the production of peptides under the conditions studied. A higher concentration of pancreatin yielded higher peptide concentrations. It was also shown that a higher peptide concentration yielded higher antioxidative properties in the hydrolysates. Therefore it can be said that a high concentration of pancreatin leads to a high concentration of antioxidative properties.

The SDS-PAGE method was shown to be useful in determining peptide molecular weights. Analysis of the SDS-PAGE results for the 19 factorial design runs showed higher digestion pancreatin concentrations to give distinctively different band patterns, in comparison to all other runs. With a high concentration of pancreatin, the high molecular weight bands (subunits of glycinin and β-conglycinin) were not present anymore. This suggests that a high pancreatin concentration helps break down larger peptides in the digestions.

The filtration of the 3 samples (+ + + +; centre point; - - - -) showed that an increase in the peptide concentration resulted in a higher flux in the 3kDa membrane. The mass balances for the 3kDa and 1kDa filtrations showed that the results for the peptide concentration and the total solids were related.

The characterisation of the peptide content after different digestions by MALDI-TOF showed that a two step digestion with pepsin and pancreatin is the most effective digestion method.

8 Perspectives

The optimisation of the filtration step should be considered as a high priority. To increase the productivity and the ease of filtration a cross flow filtration could be used instead of a stirred dead end filtration unit. This would save a lot of time due to a faster flux. Using this method fouling mechanisms could be studied as well. Another option to increase the flux could be to use a different membrane material. For example polyethersulfone is recommended for more concentrated solutions and therefore a faster flux and a higher recovery should be expected.

For the analysis of the antioxidative properties another assay should be considered as the used DPPH assay in this thesis encountered some difficulties. For example another assay, based on hydrogen atom transfer could be on option. An assay without ethanol in the solution should be chosen, because of the interaction with the proteins.

To receive a peptide distribution profile for all samples after a digestion an improved SDS-PAGE method could be used. A method to show smaller molecules (1-20kDa) would be necessary.

It would be interesting to see the amino acid composition of the different antioxidative peptides. For this the different peptides would have to be first isolated. Suggested methods for this purpose are size exclusion chromatography or RP-HPLC which separate molecules according to hydrophobicity and hydrophilicity. The molecular weight of the different peptides could then be analysed by mass spectrometry and a databases could be searched for the suggested sequences.

9 Literature

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10 Appendix

10.1 SDS-PAGE molecular weight standard

To calculate the molecular weights of the different samples on the SDS-PAGE, a marker with various known molecular weights (Table 16) was run on the same gel.

To receive a regression, the logarithms of the molecular weights of the molecules were plotted as a function of the Mobility. With this regression curve the molecular weights of the samples could be determined.

Table 16 Proteins molecular weights used as standards for the SDS-PAGE

Protein	Molecular weight [kDa]
Myosin	220
b-glactosidase	116.25
Phosphorylase b	97.4
Serum albumin	66.2
Ovalbumin	45
Carbonic anhydrase	31
Trypsin inhibitor	21.5
Lysozyme	14.4
Aprotinin	6.5

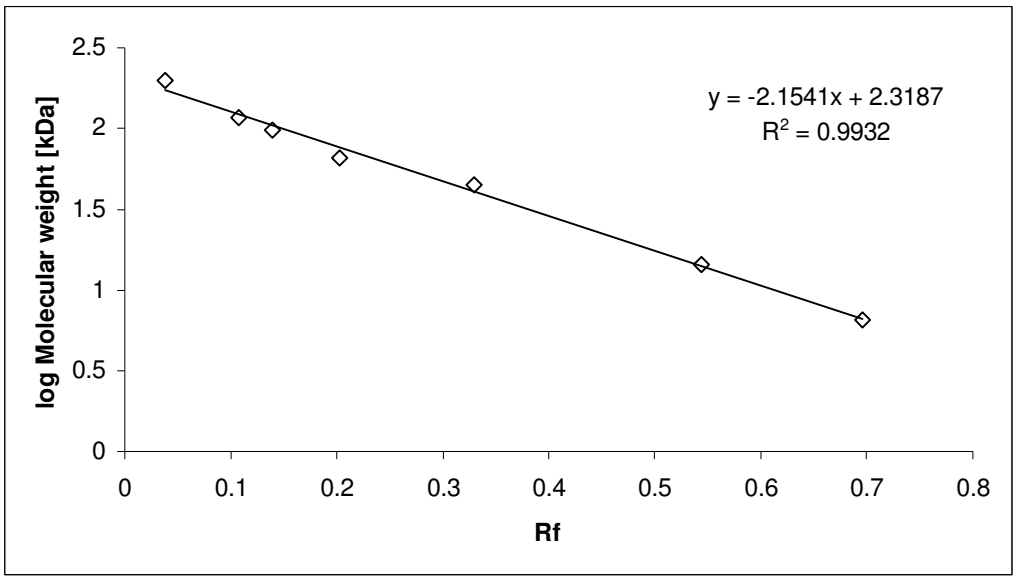

Figure 28 Molecular weight as a function of mobility for the SDS-PAGE.

10.2 OPA standard curve

The calibration curve used for the OPA assay was prepared with 5 different concentration of Phenylglycin (0 / 25 / 50 / 75 / 90µM). A good correlation factor of 0.9996 was achieved.

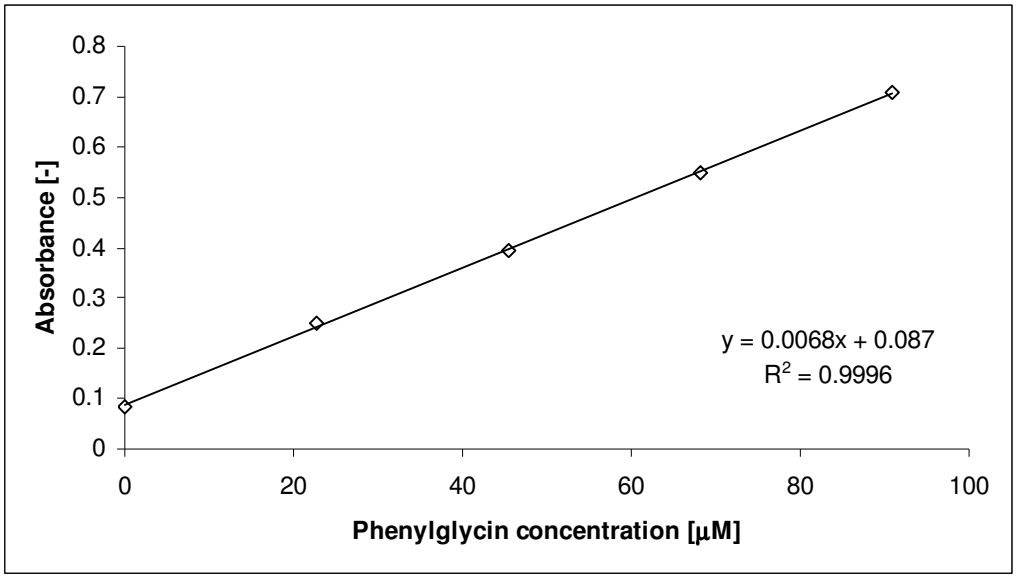

Figure 29 Phenylglycin concentration vs. absorbance. OPA assay calibration curve.

10.3 DPPH standard curve

The calibration curve for the DPPH assay was created using 5 different concentrations of ascorbic acid (0.5 / 0.4 / 0.3 / 0.2 / 0 mM). A good correlation factor of 0.9984 was achieved.

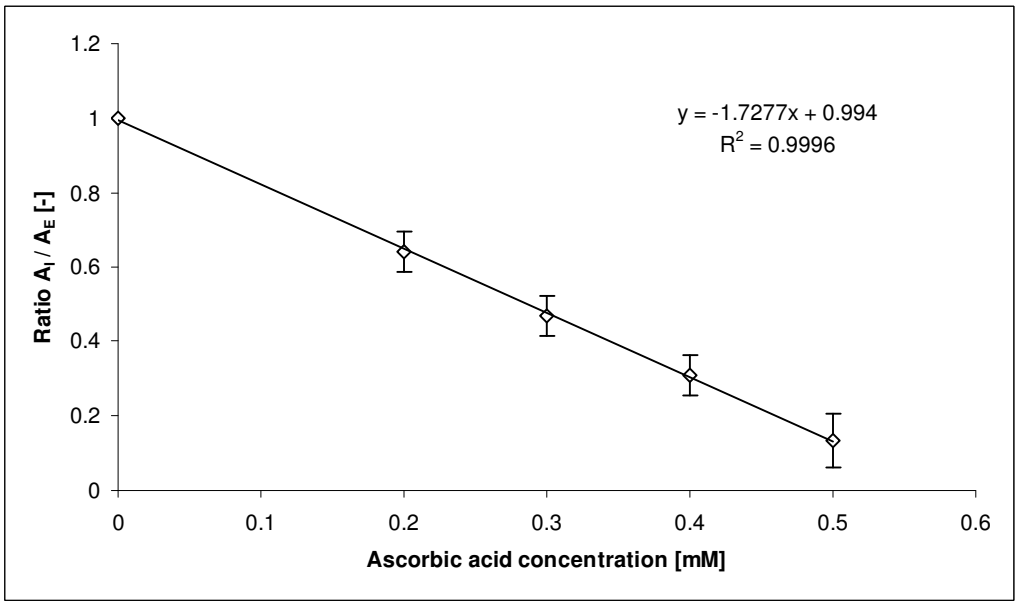

Figure 30 Calibration curve with 5 different concentrations (in triplicate) of ascorbic acid fort the DPPH assay.

10.4 Bradford calibration curve

The calibration curve used for the Bradford method was created using 6 different concentration of BSA (0 / 0.1 / 0.2 / 0.3 / 0.4 / 0.5mg/mL). A good correlation factor of 0.9933 was achieved.

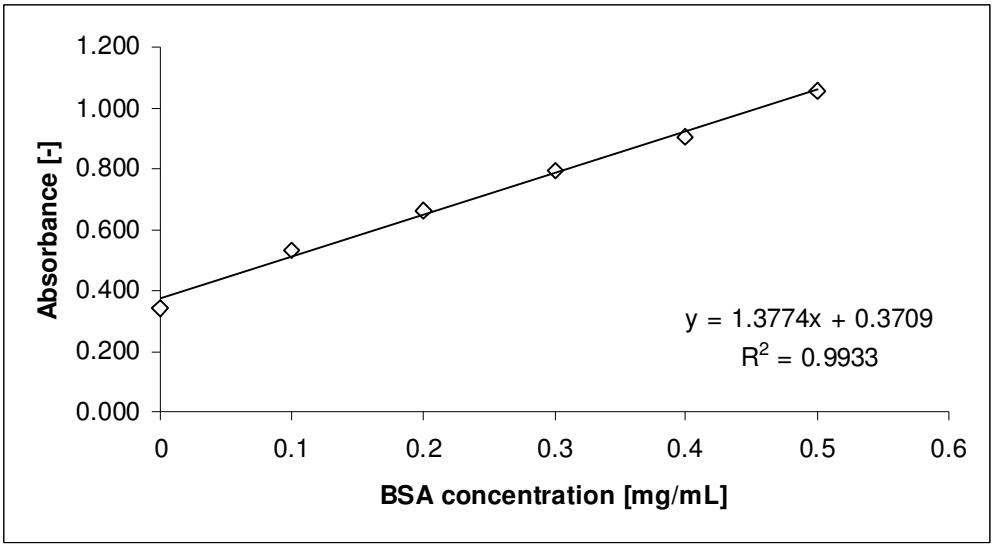

Figure 31 Calibration curve using BSA for the determination of dissolved soy protein by Bradford.

10.5 Waterflux

Before each filtration (with the 3 or 1kDa membrane) the waterflux was measured. The flux was measured at a pressure of 10 / 20 / 30 / 40 and 50 psi.

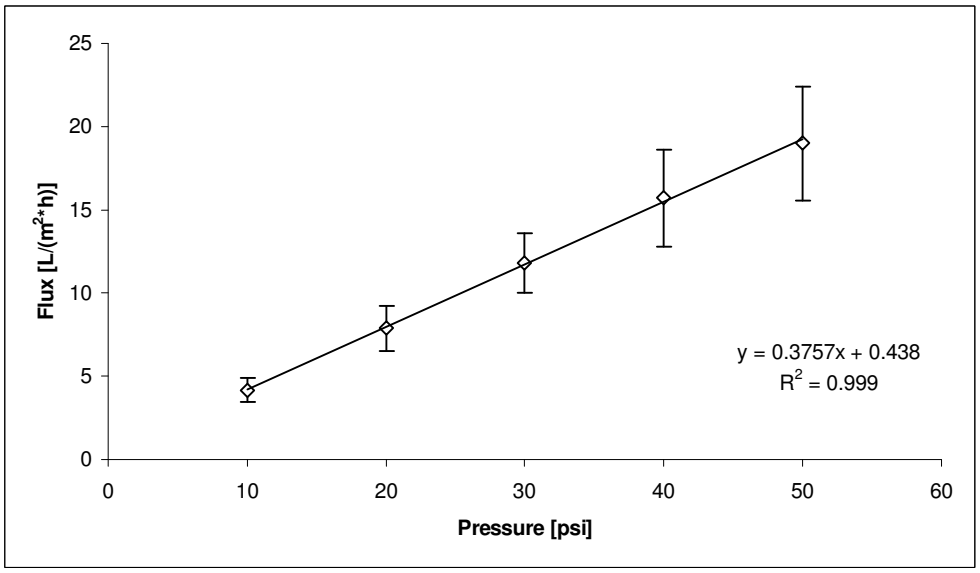

Figure 32 Four different Waterfluxes of the 1kDa membrane were performed with four different membranes (n=5)

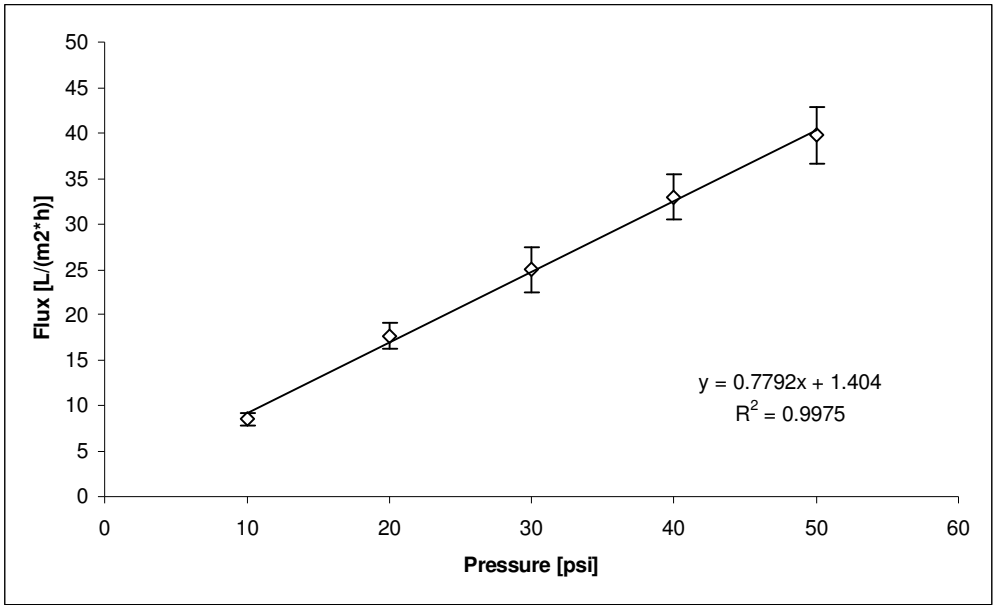

Figure 33 Four different Waterfluxes of the 3kDa membrane were performed with four different membranes (n=5)

10.6 Massbalance

The results used for analysis for the OPA assay and the total solids are shown in Tables 17-24.

Table 17 Values of the total solids used to establish the massbalance for Run 1 of the factorial design

	Total solids [g/ml]	Volume [ml]	Total solids [g]	Total solids [%]
Digestion	0.0313	150.00	4.69	100.00
YM 3 Retentate	0.0296	101.21	3.00	63.81
YM 3 Permeate	0.0242	45.53	1.10	23.47 (100)
YM 1 Retentate	0.0237	19.58	0.46	42.12
YM 1 Permeate	0.0200	14.78	0.30	26.83
YM1 membrane			0.005	0.42
YM 3 membrane			0.2	4.26

Table 18 Values of the total solids used to establish the massbalance for Run 8 of the factorial design

	Total solids [g/ml]	Volume [ml]	Total solids [g]	Total solids [%]
Digestion	0.0292	150	4.38	100.00
YM 3 Retentate	0.0304	118.24	3.59	82.07
YM 3 Permeate	0.0176	28.55	0.50	11.47 (100)
YM 1 Retentate	0.0198	10.25	0.20	40.39
YM 1 Permeate	0.0089	8.87	0.08	15.71
YM1 membrane			0.0008	0.16
YM 3 membrane			0.25	5.65

Table 19 Values of the total solids used to establish the massbalance for Run 10 of the factorial design

	Total solids [g/ml]	Volume [ml]	Total solids [g]	Total solids [%]
Digestion	0.0267	150	4.01	100.00
YM 3 Retentate	0.0296	105.83	3.13	78.22
YM 3 Permeate	0.0191	39.77	0.76	18.97 (100)
YM 1 Retentate	0.031	13.28	0.41	54.20
YM 1 Permeate	0.0119	14.11	0.17	22.11
YM1 membrane			-0.008	-1.04
YM 3 membrane			0.23	5.78

Table 20 Values of the total solids used to establish the massbalance for the filtration of the SPI sample

	Total solids [g/ml]	Volume [ml]	Total solids [g]	Total solids [%]
YM 3 Retentate	0.0402	63.28	2.54	
YM 3 Permeate	0.0162	94.14	1.53	100.00
YM 1 Retentate	0.0223	24.57	0.55	35.93
YM 1 Permeate	0.0078	53.11	0.41	27.16

Table 21 Values of the OPA assay used to establish the massbalance for Run 1 of the factorial design

	Total conc. [mmol/L]	Total amount [mmol]	Total amount [g]	Total amount [%]
Digestion	48142.6	7221.39	1091.59	100.00
YM 3 Ret	45699.9	4625.29	699.16	64.05
YM 3 Perm	46781.2	2129.95	321.96	29.50 (100)
YM 1 Ret	47658.8	933.16	141.06	43.81
YM 1 Perm	41795.8	617.74	93.38	29.00

Table 22 Values of the OPA assay used to establish the massbalance for Run 8 of the factorial design

	Total conc. [mmol/L]	Total amount [mmol]	Total amount [g]	Total amount [%]
Digestion	27487.2	4123.08	623.24	100.00
YM 3 Ret	31543.0	3729.64	563.77	90.46
YM 3 Perm	22368.2	638.61	96.53	15.49 (100)
YM 1 Ret	25939.0	265.87	40.19	41.63
YM 1 Perm	12623.2	111.98	16.93	17.53

Table 23 Values of the OPA assay used to establish the massbalance for Run 10 of the factorial design

	Total conc. [mmol/L]	Total amount [mmol]	Total amount [g]	Total amount [%]
Digestion	40318.0	6047.71	914.17	100.00
YM 3 Ret	43393.8	4592.37	694.18	75.94
YM 3 Perm	39178.2	1558.12	235.53	25.76 (100)
YM 1 Ret	43079.8	572.10	86.48	36.72
YM 1 Perm	21952.3	309.75	46.82	19.88

Table 24 Values of the OPA assay used to establishment the massbalance for the filtration of the SPI sample

	Total conc. [mmol/L]	Total amount [mmol]	Total amount [g]	Total amount [%]
Digestion	4093.95	62.02	937.5	100.00
YM 3 Ret	451.60	2.89	436.29	46.54
YM 3 Perm	457.34	0.90	136.67	14.58 (100)
YM 1 Ret	389.20	0.97	145.99	106.82
YM 1 Perm	329.42	0.89	134.88	98.69