Characterization of potato protein and its calcium binding capacity

Master Thesis in Applied Biochemistry

by

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Populärvetenskaplig sammanfatting

Kan potatisprotein öka intaget av kalcium i kroppen?

Alla vet att vitaminer och mineraler är viktiga för att kroppen ska må bra och kunna fungera. En av dom är kalcium som har stor påverkan på vår hälsa. Kalciumbrist kan ha allvarliga konsekvenser för människors hälsa och det är viktigt att få i sig tillräckligt. Kalcium i mat kan skapa vissa komplikationer, speciellt i höga halter, och kan behöva vissa hjälpmedel för att vi ska kunna uppta det i våra kroppar. En av dessa hjälpmedel finns naturligt i mjölk och det är vissa proteiner som gör att halten kalcium kan vara hög i mjölk utan att skapa dessa komplikationer när vi intar den. I denna studie undersöks potatisprotein, som är en biprodukt från produktion av potatisstärkelse, och om det kan binda in till kalcium så som vissa protein gör i mjölk. Tanken är att potatisprotein bundet till kalcium skulle kunna användas som ett tillskott i livsmedelsprodukter, framförallt växtbaserade alternativ, för att öka halten kalcium i dessa produkter.

Många känner till att kalcium har en central roll i uppbyggnaden av vårt skelett och våra tänder men kalcium är också nödvändigt för vårt nervsystem och för att skicka signaler i kroppen. Dess livsviktiga funktion betyder att kalciumbrist kan vara skadligt för vår hälsa och i längden kan det leda till benskörhet och hämma barns tillväxt. Vi upptar kalcium genom vår föda, vanligen genom att dricka mjölk, äta bladväxter eller nötter. Med framväxten av växtbaserade produkter, och framförallt växtbaserade alternativ till mjölk, finns det en osäkerhet i om vi får i oss tillräckligt med kalcium. Fördelen med mjölk som kalciumkälla är att det innehåller vissa protein, som kallas casein, som binder in till kalcium vilket gör att drycken kan innehålla en högre halt av kalcium jämfört med andra produkter. Proteinerna som binder till kalcium i mjölk formar små paket som bär kalcium genom vårt matspjälkningssystem till tunntarmen där kroppen börjar ta upp kalciumet och då släpper proteinerna ifrån sig det.

Potatisprotein består av en rad olika protein, varav en grupp heter patatin. Patatinets egenskaper tyder på att det skulle kunna binda till kalcium och potentiellt bära det genom vårt matspjälkningssystem tills vår kropp tar upp kalciumet. Potatisprotein har också många fördelar då det är en biprodukt från produktionen av potatisstärkelse, som är stor i södra Sverige, och är av lågt ekonomiskt värde då det vanligen säljs som djurfoder. Potatisprotein är en hållbar resurs och skulle kunna bidra till att förbättra andra hållbara produkter så som växtbaserade alternativ. Om potatisprotein skulle börja användas i livsmedel för människor skulle det öka värdet på resursen vilket skulle vara betydelsefullt för bönder och potatisstärkelseindustrin ekonomiskt sett.

Detta projekt går ut på att karakterisera egenskaper hos potatisprotein från en potatisstärkelseproducent och undersöka dess kapacitet för att binda till kalcium. Kalciumbindning mättes genom att mäta halten fritt kalcium i prover med protein och att använda spektroskopiska metoder för att undersöka bildning av komplex när kalcium och potatisprotein blandades. För att kunna mäta kalciumhalten tog vi fram en analysmetod som baseras på färgintensitet och spektroskopiska mätningar. Med våra metoder kunde vi bestämma innehållet i potatisproteinproverna, hur potatisproteinet löste sig i olika förhållanden och andra egenskaper som är viktiga för att förstå hur proteinet beter sig. Vi såg även bildandet av komplex i protein proverna när kalcium tillsattes och våra spektroskopiska mätningar tydde på att olika förändringar i proteinet skedde då kalcium tillsattes. Halten fritt kalcium minskade också i prover blandat med protein vilket tillsammans med de andra resultaten tyder på att potatisproteinet binder till kalcium. Det behövs en mer utökad studie på potatisproteinets kapacitet att binda till kalcium för att säga något med säkerhet men detta projekt lägger en bra grund till framtida studier med potatisprotein.

Abstract

Calcium is essential for the human body as it has functions in the nervous system, bones and formation of teeth. Deficiency in this important nutrient can be detrimental to health and cause life-long issues. Calcium can be found in various foods, like dairy products, but certain plant-based alternatives to milk needs to be fortified with calcium. One of the complications with calcium supplements is that calcium ions interact with many compounds and can become insoluble. One of the solutions to keeping calcium soluble is to form soluble complexes with proteins, as calcium does with β-casein in milk. By fortifying the calcium with proteins, a higher total concentration calcium can be attained in a solution while the free calcium concentration is still low to minimize unwanted reactions. In this thesis, potato protein, a byproduct from the potato starch production, with the potential to act as a calcium binder in plant-based products was characterized. The potato protein's potential capacity to form complexes with calcium was also assessed. The potato protein was also hydrolyzed using Alcalase in an attempt to improve its suitability as a calcium binder. The potato protein sample and hydrolysates of the proteins were analyzed with dry matter analysis and pH titration to characterize the composition, solubility and pI of the samples. Their size was investigated with gel electrophoresis and size exclusion chromatography. The calcium binding capacity was assessed by measuring free calcium concentration and monitoring changes in right-angle light scattering (RALS), absorbance at 254 nm and 280 nm, and fluorescence during calcium titration. To measure the free calcium concentration a procedure for a colorimetric calcium assay was established. Equilibrium dialysis was used when assessing the calcium binding capacity due to complications with the calcium assay. The sample of soluble potato protein (SPP) had high fraction of protease inhibitors and a lower fraction of patatin. The SPP was soluble in alkaline and highly acidic solutions, while insoluble at around pH 5. The hydrolysates had the highest insoluble fraction at pH 3, and higher soluble fractions at alkaline pH. The analysis of calcium binding capacity showed a decrease in free calcium concentration in samples with SPP and calcium, and changes in RALS, absorbance and fluorescence caused by protein aggregation and conformational changes that could indicate calcium binding. K_d-values, calculated using Hill-Langmuir equation, suggest low affinity binding. A 2^2-factorial trial indicated that pH had a significant effect on SPP-calcium binding. The experiments were also done using a well-known calcium binder, β-casein, as a positive control.

Contents

1 Introduction

Calcium is an important nutrient that we humans need for bone and teeth formation as well as blood coagulation and for our nerve system to function. Most of the calcium in the human body is found in the bones and teeth and long-term calcium deficiency can lead to brittle-bone disease or growth retardation in children. Calcium can be found in many foods, mainly dairy products, leafy vegetables and nuts. Plant-based alternatives to milk are commonly fortified with calcium [14].

Calcium interacts with many other compounds, and therefore an issue is that it can bind and precipitate as for example calcium phosphate. This is especially troublesome since phosphate is common in our bodies and calcium phosphate is relatively insoluble. A solution to this problem is that calcium can also form soluble complexes with proteins, so the free calcium concentration is maintained at a low level while the total calcium concentration in solution is higher [4]. An example of this is how the protein β-casein in milk forms large structures called micelles with calcium [6].

Plant-based products are becoming increasingly popular and it is becoming more common to replace products like cow's milk with a plant-based alternative. These alternatives are often fortified with calcium but it is as far as we know not known if there are calcium-binding proteins that can regulate the free calcium concentration or how much of the calcium in plant-based milk is actually bio-available for the consumers.

The potato starch industry produces a large amount of a protein-rich byproduct called potato fruit juice each year. This is also the case in southern Sweden where potatoes are easy and common to grow. This byproduct is mainly used for economically low-value animal feed [22]. Finding a way to use the protein from the potato fruit juice in a higher value way such as in the human diet would be beneficial both economically for potato and starch producers and sustainably. By eating the protein directly from the plant instead of feeding it to animals and consuming meat it is possible to reduce the resource use considerably. It could also be argued that eating protein directly from the plant is more ethically right.

The aim of this thesis is to characterize potato protein and explore its calcium binding capacity to see if it would be possible to use it for calcium fortification of human food. An important part of the project is to establish and validate a calcium assay that could be used to measure calcium binding. Enzymatic hydrolysis of the potato protein is also investigated and the hydrolysate is characterized to see if there are changes that could improve the calcium binding capacity.

2 Theory

2.1 Calcium

Calcium is an important cell signaling molecule. If the concentration of calcium becomes too high there can be issues such as precipitation of phosphate salt since calcium phosphate has a low solubility in water. Instead, calcium can bind to several different proteins so the free calcium ionic concentration is maintained low while still having a high total calcium concentration. Calcium can exist in ionized free form, complexed to a protein or bound to an inorganic molecule [4].

2.2 Calcium binding proteins

A remarkable feature of calcium is how it can interact in multiple ways with other molecules. As can be seen in figure 1 the main ways for calcium to form a complex with proteins is by interacting with the carboxylic acid group of peptides in the unidentate, bidentate, bridging or α-mode [1]. Many known calcium binding proteins such as calmodulin or S100 contain a tertiary structure called EF-hand with two α-helixes that interacts with the calcium ion [23]. Another way proteins can bind to calcium is by forming micelles which is the case with caseins in milk [6].

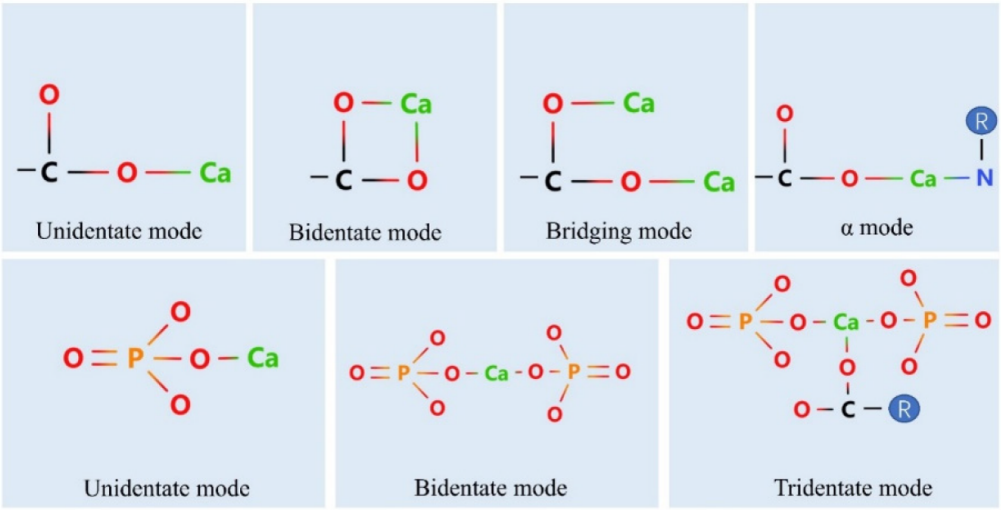


Figure 1: The different main ways for calcium interaction with proteins (top row and bottom right) and phosphate (bottom left and middle) [1].

2.3 Methods of extracting potato protein

In the potato starch industry a protein rich byproduct called potato fruit juice (PFJ) is formed. The protein in PFJ contains a large proportion of lysine and is considered as having high nutritional value. Today it is mainly used for animal feed, but there have been some attempts to use it as higher-value ingredients for human consumption. One such product is Solanic from the Netherlands-based company Avebe [2]. The most common method for extracting protein from PFJ involves heat and/or strong acid precipitation. A drawback with these methods is that the protein is often denatured and loses some of its important properties such as solubility. Other methods that can be used is salt, ethanol or ammonium sulphate precipitation, carboxyl cellulose complexation, or chromatography [22].

2.4 Potato protein

In PFJ there are mainly 3 types of proteins: patatin ($\sim$40%), protease inhibitors ($\sim$50%) and other high molecular weight proteins ($\sim$10%). Patatins have a molecular weight of 40-45 kDa and has been shown to have foaming and emulsifying abilities, as well as lipid acyl hydrolase activity. Protease inhibitors have a molecular weight of 5-25 kDa [22, 12].

2.5 O-Cresolphthalein Complexone

The ortho-cresolphthalein complexone (OCPC) is known to form a complex with calcium and is used in colorimetric methods to determine the presence and concentration of calcium in a sample. The OCPC reacts with calcium ions at alkaline conditions to form a complex with a violet color [5]. The intensity of the complex is proportional to the calcium concentration and can be measured at 570 nm [3]. When calcium ions react with OCPC it increases the acidity of ionizable protons causing their ionization and the development of color. The reaction between OCPC and calcium occurs with the calcium:OCPC ratio 1:1 and 2:1, but the absorbance of the complex seems to only be linearly proportional to the calcium concentration at the ratio 2:1. The calcium must be in an abundance in relation to OCPC to generate a linear relationship between calcium concentration and absorbance. The reaction is sensitive to temperature and pH [5].

2.6 Absorbance Spectroscopy

Absorption spectroscopy is a technique used to analyze the interaction of molecules with electromagnetic radiation. Certain molecules absorb light at different wavelengths including the peptide groups of protein main chain which absorb light in the far-UV range (180-230 nm) and the aromatic side-chains Tyrosine, Phenylalanine and Tryptophan which also absorb in the near-UV region (240-300 nm). The technique can be used for quantitative measurements by measuring the absorbance of a solute since this depends linearly on the concentration of the solute. The technique is also suitable for following protein-ligand interactions and conformational transitions in proteins as the absorbance also depends on the molecular environment of the solute's chromophores. Changes in polarity can shift the absorption spectra, an increase in polarity can decrease the absorbance slightly and shift the absorption maximum to a shorter wavelength while the absorbance of the aromatic residues is generally higher in hydrophobic environments. The absorbance of aromatic residues buried in the hydrophobic core in folded native protein can shift if their environment changes which is useful when monitoring conformational changes of a protein [18].

2.7 Fluorescence Spectroscopy

Fluorescence spectroscopy is a suitable technique for studying the formation of protein complexes. As mentioned earlier, certain aromatic side-chains in a protein absorb light but they also emit it. The excitation and emission spectra of a protein depend on the local environment of these fluorophores. The intrinsic fluorescence of a protein is dominated by tryptophan. Interactions between proteins or proteins and ligands can be monitored by measuring fluorescence since the interactions will affect the environment of the fluorophores. Through excitation at 280 nm, the changes in the emission spectra of the tryptophan and tyrosine in the protein can be measured and used to study the conformational changes of protein and complex formation [9].

2.8 Right-Angle Light Scattering (RALS)

Static Light Scattering can be used to follow the aggregation of small molecules and macromolecules like proteins in solution. If the particles are smaller than the light wavelength, the light scattering follows the Rayleigh theory, and the degree of aggregation is proportional to the fraction of scattered light. The right angle light scattering (RALS) is

static light scattering recorded by a detector at 90°. This technique, compared to forward light scattering, gives an advantage in detection of smaller changes [15].

2.9 Hill-Langmuir

The Hill-Langmuir equation for equilibrium binding is based on the assumption of random interaction between ligands and the receivers. The response is driven by a function of concentration and affinity. The Hill-Langmuir equation, equation 1, can be used to estimate the protein-ligand affinity or rather dissociation. In this study the receivers are the potato proteins and the ligand is calcium.

$$\theta = \frac{[L]^n}{K_D + [L]^n} \tag{1}$$

The θ is the fraction of receptor concentration that is bound by the ligand, $[L]$ is the total ligand concentration, n is the Hill coefficient and K_D is the apparent equilibrium dissociation constant. The Hill coefficient is a dimensionless, macroscopic parameter that measures cooperativity of ligands in a binding process. The assumptions for these equations are that all the receptors are equally accessible to the ligands, the binding is reversible and the receptors are either free or bound to the ligand, no partial binding [7].

2.10 Equilibrium Dialysis

Equilibrium dialysis is a simple, inexpensive, and direct method to determine binding constants between proteins and ligands. The setup is two small chambers separated by a dialysis membrane with a molecular weight cut-off below the weight of the protein but that allows the ligand to diffuse through. Protein and ligand are in buffers and added to a chamber. The ligand will diffuse through the membrane to the other chamber until it reaches equilibrium but the protein will only stay in one of the chambers where it binds the ligand [11]. The method is suitable when the protein interferes with the quantification method of the ligand. By taking a sample from the chamber without the protein, the concentration of the ligand can be measured without the interference of protein in the quantification method. The principle of equilibrium is shown in figure 2.

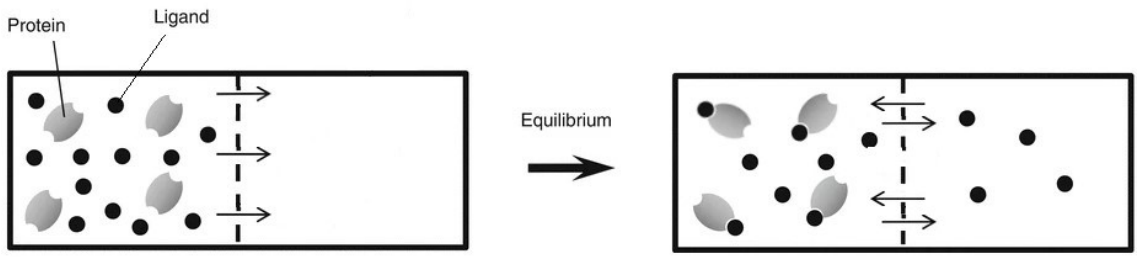


Figure 2: Illustration of equilibrium dialysis with protein and ligand.

2.11 Isoelectric point of proteins

The isoelectric point (pI) of proteins is important in understanding protein solubility and protein-ligand interactions. Proteins can have different net charges due to the charges of the carboxyl and amino groups that are part of its structure. The net charge of a protein varies with pH as the charges of these groups depend on pH. The isoelectric point of a protein is the pH at which the net charge of the protein is zero. The net charge is positive

at pH below the pI and negative at pH greater than the pI [16]. The solubility of a protein is affected by the pH due to the effect it has on the net charge. The proteins will repel each other if they have similar charges but at the pI the net charge is zero and the proteins are no longer kept separate due to the low repulsive forces of the electrostatic interactions at this pH. At the pI, attractive interactions can dominate which tends to lead to protein aggregation and the large aggregates can be insoluble [21]. The charges of a protein are also important for protein-ligand interactions. Both protein and ligand should not be both positively charged or negatively charged since they will repel each other. It is important to know the pI of a protein and be familiar with the state of the protein over a range of pH, in order to understand and promote protein-ligand interactions [16].

2.12 SDS-PAGE

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) is an analytical method to separate proteins based on their mass. The samples are treated with heat and the detergent sodium docedyl sulphate (SDS) to unfold and charge the proteins. A reducing agent can also be used to break disulphide bonds. After that samples are loaded at the top of a polyacrylamide gel. Voltage is applied over the gel to make the charged particles migrate through the gel. Larger proteins will migrate slower and shorter while smaller proteins can migrate faster and longer [17, 13].

2.13 HPLC-SEC

Size exclusion chromatography (SEC) is a method to separate particles based on size by letting them pass though a column packed with a porous material. Smaller particles can go inside the pores of the material which will give them a longer path making it take longer to go through the column compared to larger particles that do not enter the pores of the material [19].

High-perfomance liquid chromatography (HPLC) is a chromatography method where a liquid is passed through the column using a flow with high pressure, so that it is possible to separate compounds of interest based on their properties such as size in SEC [10].

2.14 Hydrolysis

The action of breaking the peptide bonds in proteins to form shorter peptides or single amino acids is called hydrolysis. One way to facilitate hydrolysis is through using enzymes called proteases, the proteases will facilitate the cleaving of peptide bonds. Proteases are classified as EC 3.4 meaning hydrolases that aids hydrolysis of peptide bonds. Endopeptidases cleaves in the middle of the amino acid chain while exopeptidases cleaves of 1-3 amino acid long fragments from the ends [20].

2.14.1 Alcalase

Alcalse is an enzyme from *Bacillus Subtilis* with hydrolytic activity. It is a serine endopeptidase, meaning it cleaves peptides in the middle of the peptide chain and has a serine in the catalytic site. It has a broad selectivity and has been described to prefer cleaving to the right of a large uncharged residue or Glu, Met, Leu, Tyr, Lys, and Gln. It has also been described to prefer cleaving to the right of Glu when there is a hydrophobic amino acid at the second and third position to the right of the cleavage site. With this broad selectivity Alcalase is expected to produce many small peptide fragments [20].

3 Method

3.1 Stock solution of SPP

To prepare a stock solution of soluble potato protein (SPP), 2g SPP powder (Gifted from Lyckeby, Sweden) was mixed with 40 ml Milli-Q water (50 mg/ml) in a 50 ml falcon tube. The solution was mixed for 24 h at 4°C to fully hydrate the protein. As a validation, pH was measured to ensure it was always pH 7-8. The solution was then centrifuged at 9000rpm (11139g) for 10 min. The supernatant was transferred to a new tube, stored in the fridge for up to 60 days and used as SPP stock solution. To ensure a constant concentration a dry matter analysis was performed on each newly prepared stock solution. The concentration of soluble protein in the stock was estimated to 27 mg/ml.

3.2 Calcium Assay

3.2.1 Standard Curve

The calcium assay was performed on a 96-well plate and used to determine the free calcium concentration in a sample. Standard curves were established using 0.1 M HCl in Milli-Q water or 10 mM Tris-HCl, pH 8.5, as solvents. A stock solution of $CaCl_2$ was prepared with the calcium concentration 300 mg/l in 0.1 M HCl and one with 800 mg/l in 10 mM Tris-HCl, pH 8.5. For the standard curve in 0.1 M HCl, three dilution series of $CaCl_2$ standard solutions were done with calcium concentrations at 1, 2, 3, 4, 5 and 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 50, 100, 150, 200 and 250 mg/l. For the standard curve in 10 mM Tris-HCl, a dilution series of $CaCl_2$ standard solutions was done with calcium concentrations at 5, 10, 50, 100, 200 and 250 mg/l. O-cresolphthalein was dissolved in 0.2 M HCl to make 75 mg/l o-cresolphthalein stock solution (OCPC). 5 µl of blank or calcium standard was added to each well, triplicates were taken of each. The blank was either Milli-Q water or 10 mM Tris-HCl. 190 µl of 1 M Tris-HCl buffer, pH 9.4, was added to each well and mixed with a pipette. 5 minutes after mixing, 50 µl of OCPC was added to each well and mixed well with a pipette. The samples in the 96-well plate were analyzed by measuring absorbance with a spectrophotometer (SPECTROstar Nano, BMG LABTECH, Germany) at wavelengths 570 nm (main) and 700 nm (sub). The protocol with the settings for the spectrophotometric measurements are included in Appendix A. The measuremnets were done at 5, 10, 15 and 20 min. All reagents were at room temperature during use.

The absorbance was corrected according to equation 7 and the standard curve was made with linear regression based on the standards according to equation 8 in Appendix C.

3.2.2 Samples

Calcium assay was done using a blank, two calcium standards and the samples on the same 96-well plate. The blank was either Milli-Q water or 10 mM Tris-HCl, depending on the solvent the samples were in. 5 µl of blank, calcium standard or sample was added to the wells, triplicates were taken of each. 190 µl of 1 M Tris-HCl buffer, pH 9.4, was added to each well and mixed with a pipette. 5 minutes after mixing, 50 µl of OCPC was added to each well and mixed with a pipette. The samples in the 96-well plate were analyzed by measuring absorbance with a spectrophotometer (SPECTROstar Nano, BMG LABTECH, Germany) at wavelengths 570 nm (main) and 700 nm (sub). The protocol with the settings for the spectrophotometric measurements are included in Appendix A. The measurement was made after 20 min. All reagents were at room temperature during use.

The absorbance was corrected according to equation 7, shown in Appendix C. The calcium concentration was calculated by making a linear regression based on the calcium standards and using equations 8 and 9.

3.3 Kinetic Calcium Assay

Kinetic Calcium Assay was done to measure the binding of OCPC to calcium over time and how it affected the protein binding. Samples of protein and calcium were prepared 24h or just before analysis. The samples contained 1 mg/ml protein, either SPP or β-casein (from bovine milk, Sigma-Aldrich), and 250 mg/l calcium. The SPP stock solution in Milli-Q water was used and diluted with 10 mM Tris-HCl, pH 8.5. β-casein stock solution (10 mg/ml) in 10 mM Tris-HCl, pH 8.5, was prepared. A control for each protein, SPP and β-casein, was used containing the protein and no calcium. The samples prepared 24h prior to analysis were kept at 5°C with no agitation and then gently mixed before samples for the calcium assay were taken. The 96-well plate for Kinetic Calcium Assay was prepared as previously described in the section ”Samples” in ”Calcium Assay” and the optical density (OD) was corrected the same way too. The protocol with the settings for the spectrophotometric measurements for the Kinetic Calcium Assay are in Appendix A. All reagents were at room temperature during use.

3.4 Equilibrium Dialysis

3.4.1 Establishing Equilibration Time

5 units of SpinDialyzer™ (Harvard Apparatus) were used for equilibrium dialysis, an illustration of the unit is in figure 24 in Appendix D. The SpinDialyzer™ was not use as intended, as they were not spun around, but instead a magnetic stirring bar was used. The SpinDialyzers™ were cleaned in 1 L 0.5 M NaOH for 30 min then rinsed with Milli-Q water and scrubbed with Q-tips. To each bottom chamber a magnetic stirring bar was added and then filled with a total of 980 µl of sample. The bottom sample consisted of $CaCl_2$ Solution in 10 mM Tris-HCl which would result in a total calcium concentration of 100 mg/l. The dialysis membrane, a cellulose acetate membrane (MWCO 1kDa, Harvard Apparatus, USA), was then rinsed in Milli-Q water before being placed in the SpinDialyzer™, using a metal tweezer. The top part of the SpinDialyzer™ was screwed on and 980 µl 10 mM Tris-HCl buffer was added in the top chamber. The SpinDialyzers™ were sealed with parafilm. The units were placed on magnetic stirring plates at stirring speed 500 rpm and room temperature, approximately 20°C. The samples for calcium assay analysis were taken in triplicates from the top chamber after 24h, 48h, 72h and 96h. Samples that were not analyzed the same day were stored overnight in a sealed 96-well plate at 5°C. The calcium assay was performed according to the section ”Samples” in ”Calcium Assay”. All reagents were at room temperature during use.

3.4.2 2^2-Factorial Trial

In the 2^2-factorial trial two different protein concentrations and two different pH were tested (table 1). The two different protein concentrations were 5 mg/ml and 10 mg/ml of SPP which were diluted in either 10 mM Tris-HCl buffer, pH 8.5, or in 0.1 M Tris-HCl buffer, pH 6.8. The SPP stock solution in Milli-Q water was used. 5 units of SpinDialyzer™ (Harvard Apparatus) were used for equilibrium dialysis, an illustration of the unit is in figure 24 in Appendix D. The SpinDialyzer™ was not use as intended, as they were not spun around, but instead a magnetic stirring bar was used. To each bottom chamber a magnetic

stirring bar was added and then filled with a total of 980 µl of sample. The sample consisted of SPP (either 5 mg/ml or 10 mg/ml), diluted in Tris-HCl buffer (either pH 8.5 or pH 6.8) and $CaCl_2$ solution, diluted in Tris-HCl buffer (either pH 8.5 or pH 6.8). The total calcium concentration at equilibrium would be 100 mg/l. One of the units was a control without protein. The dialysis membrane, a cellulose acetate membrane (MWCO 1kDa, Harvard Apparatus, USA), was then rinsed in Milli-Q water before placed in the SpinDialyzer™, using a metal tweezer. The top part of the SpinDialyzer™ was screwed on and 980 µl Tris-HCl buffer, either pH 8.5 or pH 6.8, was added in the top chamber. The SpinDialyzers™ were sealed with parafilm. The units were placed on magnetic stirring plates for 72h at stirring speed 500 rpm and room temperature, approximately 20°C. The samples for calcium assay analysis were taken in triplicates from the top chamber which was performed according to the section ”Samples” in ”Calcium Assay”. All reagents were at room temperature during use.

Table 1: The factors and combination of them used in the 2^2-factorial trial. The protein used was SPP. The order of the samples was randomized.

Sample ID	pH	Protein Concentration [mg/ml]
1	6.8	5
2	8.5	5
3	6.8	10
4	8.5	10

A statistical analysis was used to assess the data from the trial using Jamovi with General Linear Model. The results were analyzed using flexplot and the ANOVA of the model.

3.4.3 Binding Curve

5 units of SpinDialyzer™ (Harvard Apparatus) were used for the equilibrium dialysis, an illustration of the unit is in figure 24 in Appendix D. The SpinDialyzer™ was not use as intended, as they were not spun around, but instead a magnetic stirring bar was used. To each bottom chamber a magnetic stirring bar was added and then filled with a total of 980 µl of sample. The bottom sample consisted of $CaCl_2$ solution in 10 mM Tris-HCl and of protein (5 mg/ml), either SPP or β-casein (from Bovine Milk), in 10 mM Tris-HCl. For the binding curve with SPP, $CaCl_2$ solution in 10 mM Tris-HCl was added to get the total calcium concentrations 25, 50, 100, 150 and 200 mg/l calcium. For the binding curve with β-casein, $CaCl_2$ solution in 10 mM Tris-HCl was added to get the total calcium concentrations 50, 100, 150, 200 and 250 mg/l. The dialysis membrane, a cellulose acetate membrane (MWCO 1kDa, Harvard Apparatus, USA), was then rinsed in Milli-Q water before being placed in the SpinDialyzer™, using a metal tweezer. The top part of the SpinDialyzer™ was screwed on and 980µl 10 mM Tris-HCl buffer was added in the top chamber. The SpinDialyzers™ were sealed with parafilm. The units were placed on magnetic stirring plates for 72h at stirring speed 500 rpm and room temperature, approximately 20°C. The samples for calcium assay analysis were taken in triplicates from the top chamber. All reagents were at room temperature during use.

3.5 Hydrolysis

Hydrolysates were prepared as described by Gregersen Echers et al. [8] with some modifications. pH was measured but not adjusted. SPP was heated at 90°C for 30 minutes to

inactivate protease inhibitors. The resulting slurry was diluted with Milli-Q water to get a more liquid sample and the pH was measured. Then Alcalase® (2.4 L FG, Novozymes, Denmark) was added and samples were kept at 50°C for 2 hours for hydrolysis. pH was measured again and samples were heated at 90°C for 15 minutes to inactivate the Alcalase. Samples were then cooled down and centrifuged at 10300rpm (ca 10.082g) for 20 minutes. The supernatant was collected and stored cold until analysis for up to 10 days.

For the hydrolysis, an experimental design was done using two different dilutions of the slurry and three different enzyme concentrations as can be seen in table 2. The dilution was calculated based on the total dilution from the initial volume of SPP stock to the final volume of hydrolysate. The enzyme-to-substrate (E:S) ratio is based on the added weight of Alcalase stock with the assumption that 1ml=1g and the weight of SPP as 50 mg/ml stock. More details on the added volumes are found in Appendix B in table 11. The experimental design was done to find out if the dilution and enzyme-to-substrate ratio had an effect on the degree of hydrolysis and characteristics including the calcium binding capacity of the hydrolysates.

Table 2: Experimental design of the hydrolysis. For the different E:S ratio levels the amount of added enzyme is 50 μl (high), 10 μl (mid) and 5 μl (low) which results in slightly different E:S ratios in the different dilutions since the amount of added SPP is different.

Sample ID	E:S ratio [mg:mg]	Dilution factor
A	1:8 (High)	2x (High)
B	1:38 (Mid)	2x
C	1:75 (Low)	2x
D	1:10 (High)	1.33x (Low)
E	1:50 (Mid)	1.33x
F	1:100 (Low)	1.33x

3.6 SDS-PAGE

To analyze protein content of samples SDS-PAGE was used. Samples were diluted in wash buffer or Milli-Q water (10x-100x final dilution) to a volume of 40 µl and 40 µl sample buffer was added to get a 2x dilution of the sample buffer. Then they were heated at 85 °C for 10 minutes to denature the proteins. Samples were cooled for a few minutes before 5 µl was loaded into the gel. 10 µl of ladder was used. 90 V voltage was applied over the gel for 10 minutes and 200 V until the bands had moved through. The gel was washed in deionized (di) water, stained with safe stain and destained in di water overnight. Then a photo of the gel was taken and analyzed in ImageJ.

3.7 HPLC-SEC

Samples were also analyzed with High performance liquid chromatography (HPLC) (Agilent 1100, Agilent Technologies) using a size exclusion chromatography column (SEC) TSKgel® G3000SW$_{XL}$ (Tosoh, Japan) to quantify the protein content and better understand the presence of protein sizes in their native state in the samples. Samples were either ran undiluted or diluted using running buffer (Tris, pH 9, 100 nM NaCl).

3.8 pH Titration

The absorbance, pH and RALS were measured in protein samples during titration of 0.5 M NaOH in Milli-Q water. The protein samples were acidified with 1 M HCl to pH 2-2.5 and stored at 5°C overnight. The pH electrode (Orion Star™ A215, Thermo Fisher Scientific) was calibrated with 3 buffers at pH 4.01, 7.00 and 9.21. The automatic syringe on the Labbot (labbot, Probation Labs Sweden AB, Sweden) was washed with the titrant by loading and ejecting the titrant 3 times before filling it with 100 µl. The spectrophotometer was prepared with 2 ml Milli-Q water as blank. 2 ml protein sample was added to a quartz cuvette (Suprasil®, Hellma) and placed in the Labbot. A volumetric concentration titration was done according to the instructions in the Labbot manual. 0.5 M NaOH solution was titrated in steps of 2.5 µl while continuously stirring at speed 6. The equilibration time after each injection was 30 seconds and the temperature control was set at 20°C. Record Electrode was on to record the pH. The absorbance was recorded between the wavelengths 250 - 770 nm with four averages. The Labbot procedure is in Appendix A. All reagents were at room temperature during use.

The titration was done with 1 mg/ml SPP diluted in Milli-Q water which was prepared from SPP stock solution in Milli-Q water, with the estimated concentration 27 mg/ml. The procedure was repeated with SPP hydrolysate samples.

3.9 Calcium Titration

The absorbance, fluorescence and RALS were measured in a protein sample while $CaCl_2$ solution was titrated down in it using a Labbot (labbot, Probation Labs Sweden AB, Sweden). The titrant was $CaCl_2$ solution with 1 M calcium in 10 mM Tris-HCl buffer, pH 8.5. The protein sample consisted of 1 mg/ml SPP diluted in 10 mM Tris-HCl buffer, pH 8.5. The pH electrode (Orion Star™ A215, Thermo Fisher Scientific) was calibrated with 3 buffers at pH 4.01, 7.00 and 9.21. The automatic syringe on the Labbot was washed with the titrant by loading and ejecting the titrant 3 times before filling it with 100 µl. The spectrophotometer was prepared with 3 ml Milli-Q water as blank. 3 ml protein sample was added to a quartz cuvette (Suprasil®, Hellma) and placed in the Labbot. A volumetric concentration titration was done according to the instructions in the Labbot manual. 1 M calcium solution was titrated in steps of 2.5 µl while continuously stirring at speed 6. The equilibration time after each injection was 60 seconds and the temperature control was set at 20°C. Record Electrode was on to record the pH. More information about the Labbot protocol can be found in Appendix A. All reagents were at room temperature during use.

The procedure was repeated with a control containing only 10mM Tris and no protein. The procedure was repeated as above but with 1 mg/ml β-casein instead of SPP. The calcium titration was performed as described above with SPP hydrolysate samples.

The relative RALS was calulated from the RALS data by dividing it with the maximum RALS value and then fitted with the Hill-Langmuir equation, equation 1, to calculate the binding constants.

3.10 Dry Matter Analysis

For each sample 3 aluminium boats were prepared using aluminium foil to get 3 replicates. 1 ml of supernatant was added into each boat, the rest of the supernatant stored for further use or discarded. Aluminium boats and tubes containing the pellet were then left to dry on the lab bench for 17-65 hours and then placed in the oven at 100 °C for 2 hours to be completely dry. The aluminium boats were weighed before adding sample (m_{foil}), after adding sample and after drying ($m_{foil+supernatant,dry}$). The tubes containing pellet

were weighed after drying ($m_{tube+pellet,dry}$) and then after removing the pellet (m_{tube}). The measurements were used to calculate the dry matter of the pellet (dm_{pellet}) and supernatant ($dm_{supernatant}$) as well as the soluble (S) and insoluble (I) fraction as:

$$dm_{pellet} = m_{tube+pellet,dry} - m_{tube} \tag{2}$$

$$dm_{supernatant} = m_{foil+supernatant,dry} - m_{foil} \tag{3}$$

$$m_{total} = C_{sample} \cdot V_{sample} \tag{4}$$

$$S = \frac{dm_{supernatant}}{m_{total}} \tag{5}$$

$$I = \frac{dm_{pellet}}{m_{total}} \tag{6}$$

4 Results

4.1 SPP characterization

4.1.1 pH titration of SPP

After some tuning of the pH titration with SPP it was done successfully. It could be seen that the titration curve increased most around pH 5 and 8.5 (fig. 3). When looking at the RALS there was some RALS at acidic conditions, it then increased with a peak around pH 5, and stabilized at a low RALS at alkaline conditions (fig. 4).

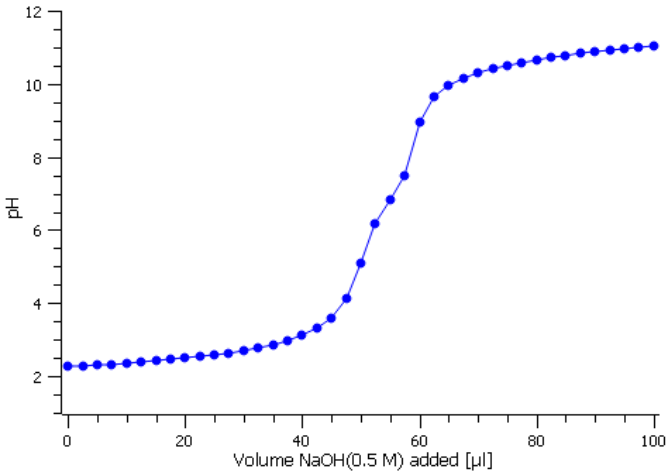


Figure 3: pH titration curve for soluble potato protein (SPP).

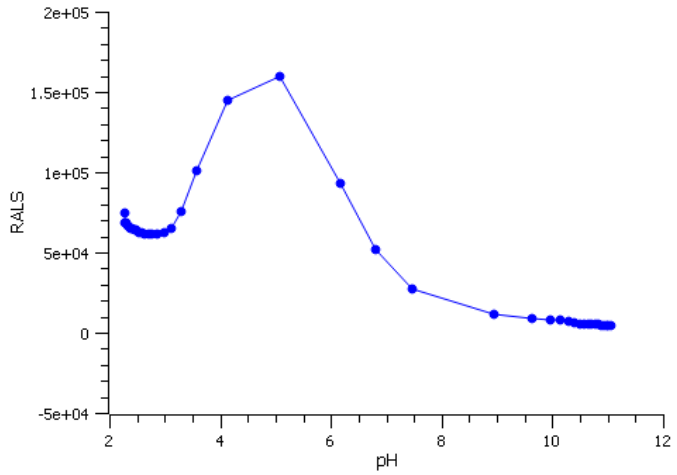


Figure 4: Right-angle light scattering (RALS) during pH titration of soluble potato protein (SPP).

4.1.2 Dry matter analysis of SPP solubility

The dry matter analysis solubility varied slightly (51.7-56.8 %) between stocks, with an average of 54 % for stocks 1-3 (table 3). Therefore 54 % was used to estimate an average of 27 mg/ml protein in all stock preparations in further calculations on protein concentrations and dilutions.

Table 3: Dry matter analysis results of SPP stock solubilty.

Date	Stock ID	Solubility (%)	Standard error
2023-03-01	A	54.7	0.30
2023-03-10	B	55.8	0.33
2023-04-25	C	51.7	0.20
2023-04-25	D	56.8	0.25
2023-05-03	E	52.6	0.16
2023-05-03	F	52.3	0.14

The dry matter analysis at different pH with both the SPP and the food-grade coagulated potato protein (FPP) from Lyckeby showed a higher overall solubility of the SPP. SPP has a lower solubility at pH 5 and higher solubility at the most alkaline pH studied (pH 10). FPP generally has a low solubility of less than 20 % (fig. 5).

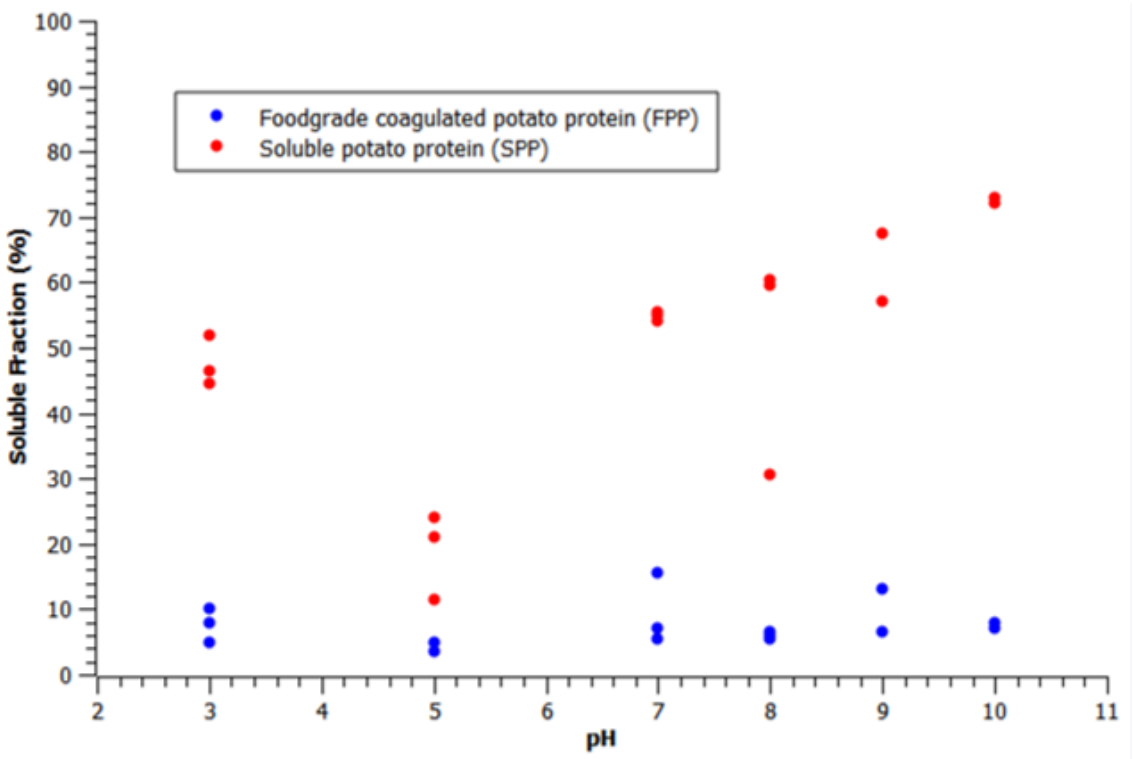


Figure 5: Dry matter analysis results of potato protein solubility at different pH. SPP in red and the food-grade coagulated potato protein from Lyckeby in blue.

4.1.3 SDS-PAGE of SPP

Samples were taken during SPP stock preparation according to table 4. Analysis with SDS-PAGE showed a relatively similar protein size content of the different samples. The pellet has a thinner band around 10 kDa compared to samples before centrifugation and the supernatant, while supernatant has a somewhat smaller band around 40 kDa compared to the pellet and the solution before centrifugation. The bands around 20 kDa are strong in all samples (fig. 6).

Table 4: Samples taken during SPP stock preparation for SDS-PAGE analysis.

Sample ID	Sample	Dilution factor
A	Before centrifugation	100x
B	Supernatant	50x
C	Supernatant	100x
D	Pellet	100x
E	Between pellet and supernatant	100x

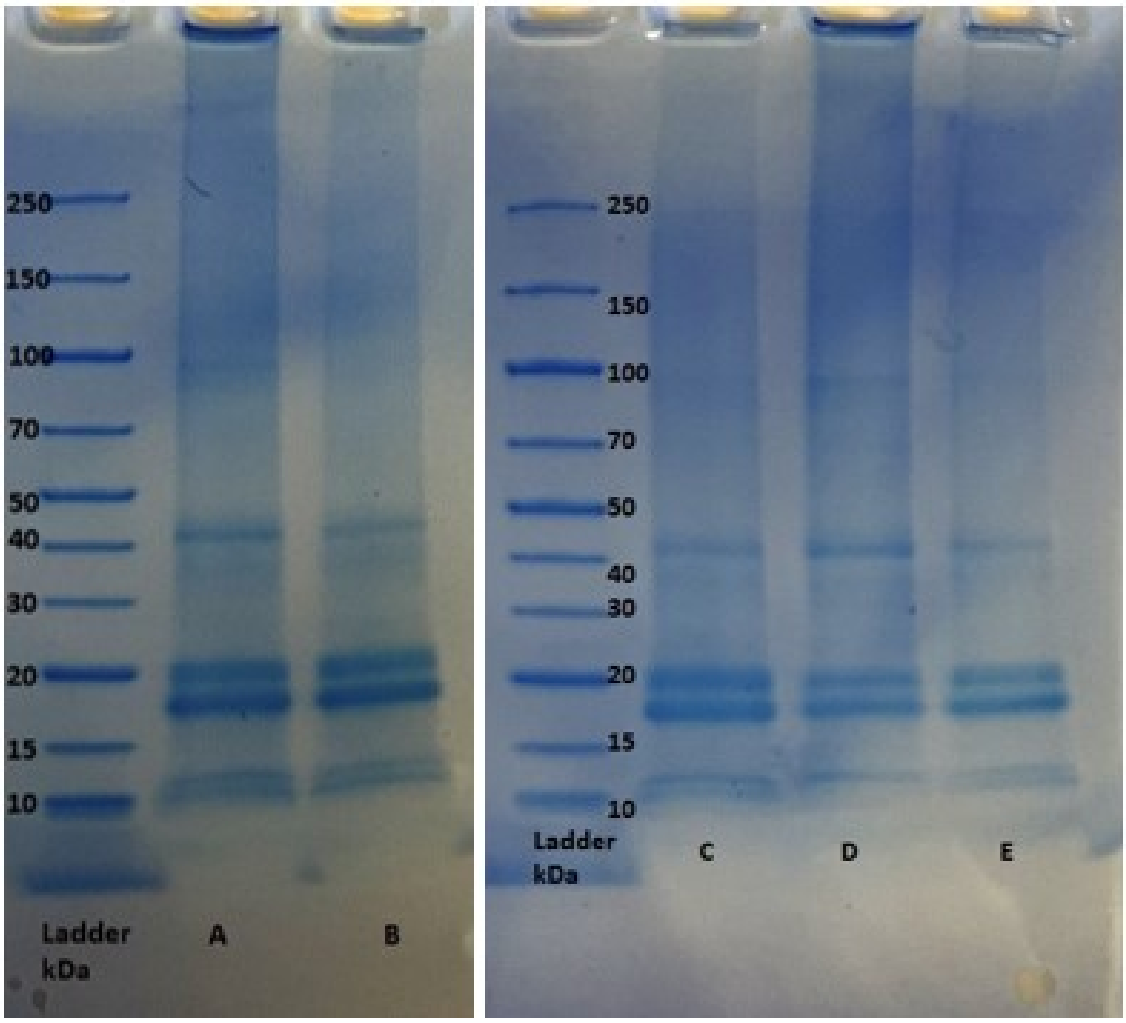


Figure 6: SDS-PAGE results of the different SPP samples A-E, A-B on the gel to the left and C-E on the gel to the right. A detailed sample description in table 4.

4.2 Calcium Assay

4.2.1 Establishing Calcium Assay

The first part of establishing the calcium assay was to establish the protocol for the spectrophotometer by testing different well scans and incubation times. The results showed different well scans did not affect the linearity or the span of the error bars, but the incubation times did. The error bars were smaller after 10-20 min and the increase in absorbance abated after 10 min and stabilized around 20 min. The results are shown in Appendix E. The linear regression based on the OD measurements on the standards in 0.1 M HCl are shown in figure 7. The R^2 of the linear regression is 0.998 which shows a high goodness of fit. The slope in figure 7 was $0.00308 \pm 2.15 \cdot 10^{-5}$ and the intercept was -0.0112 ± 0.00184.

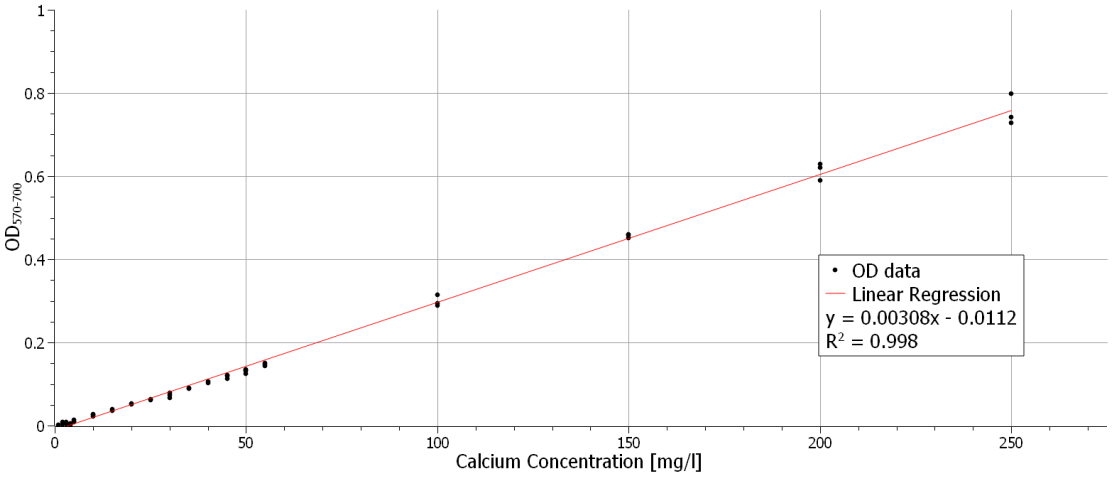


Figure 7: Linear regression of OD measurements on calcium standards 1-250 mg/l calcium in 0.1 M HCl. The equation and goodness of fit for the linear regression is displayed in the graph.

The linear regression based on the standards in 10 mM Tris-HCl, pH 8.5, are shown in figure 8. The goodness of fit is similar to the standards in 0.1 M HCl but the OD values are lower. The error bars of the replicates have over all diminished, especially in the high OD region but the error in the slope have increased. The slope of the regression in figure 8 was 0.00171 $\pm 2.64 \cdot 10^{-5}$ and the intercept was -2.10·10^{-5} $\pm$ 0.00365.

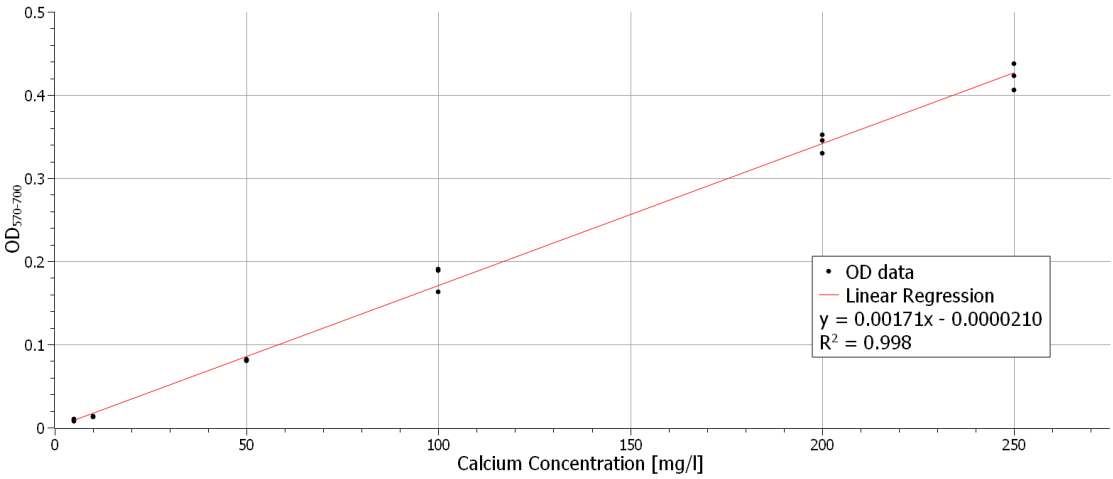


Figure 8: Linear regression of OD measurements on calcium standards 1-250 mg/l calcium in 10 mM Tris-HCl. The equation and goodness of fit for the linear regression is displayed in the graph.

The known calcium binding protein, β-casein, was used to validate if the calcium assay could be used to measure calcium binding. The results from direct sampling from mixed calcium and β-casein are shown in figure 9. The measured calcium concentration in the samples were about the same as the added calcium concentration.

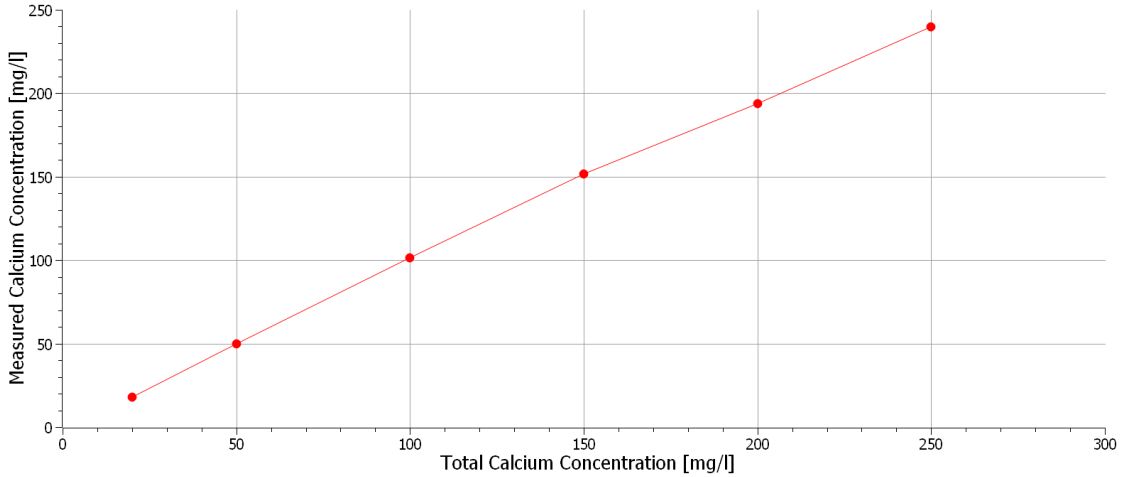


Figure 9: The measured free calcium in samples with 5 mg/ml (0.21 mM) β-casein in 10 mM Tris-HCl. The calcium and protein samples were mixed and shortly after analyzed with the calcium assay.

4.2.2 Equilibrium Dialysis

Equilibrium dialysis was used to separate where the protein was and where the calcium was sampled. The results to establish the equilibration time for calcium in equilibrium dialysis are presented in Appendix E. The calcium concentrations were calculated from the results obtained from dialysis with calcium and β-casein, and are presented in figure 10. These results show a higher calcium binding than previous measurements with direct sampling from calcium-β-casein mix. The data point at 250 mg/l was assumed to be an outlier.

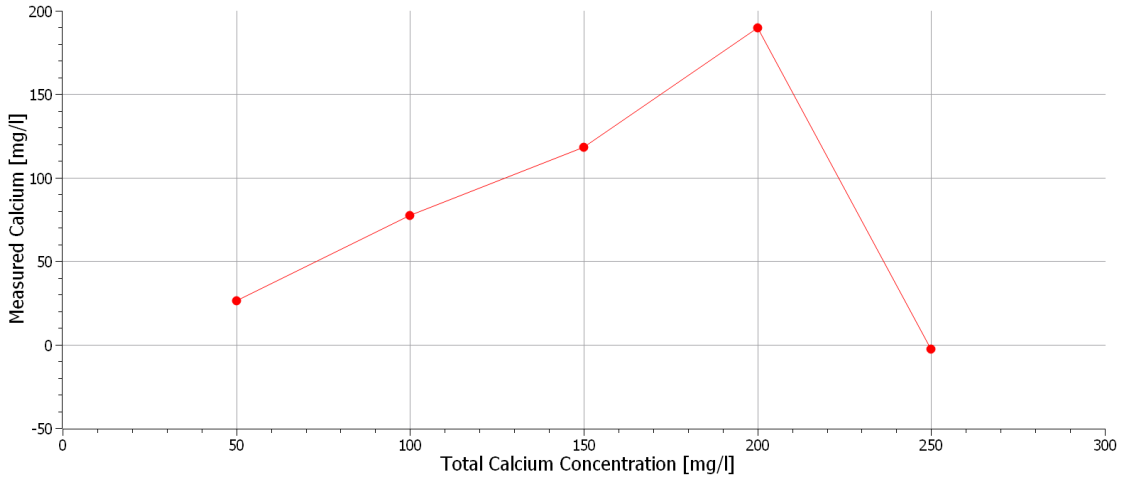


Figure 10: The measured free calcium in dialysis samples with 5 mg/ml (0.21 mM) β-casein in 10 mM Tris-HCl. Calcium and protein was mixed in the dialysis units for 72h before analyzing samples with the calcium assay.

A 2^2-factorial trial was done to assess the effect of pH and protein concentration on calcium-SPP binding. The calculated calcium concentration of the results are presented in figure 11. A General Linear Model (GLM) was done based on the trial to assess the effect of the factors. The flexplot, from GLM, of the OD results in figure 12 show the effect of the factors, protein concentration and pH, on the binding. The effect of protein concentrations is lower at pH 8.5 compared to pH 6.8. The absorbance is highest for

protein concentration 5 mg/ml at pH 6.8 which means there is more free calcium in the sample while the lowest free calcium concentration is at pH 8.5 with 10 mg/ml SPP.

Table 5: The results of the 2^2-factorial trial. The order of the samples was randomized. The response is the average calculated free calcium concentration in the sample.

Sample ID	pH	Protein Concentration [mg/ml]	Response [mg/l]
1	6.8	5	59.3
2	8.5	5	15.1
3	6.8	10	20.5
4	8.5	10	12.6

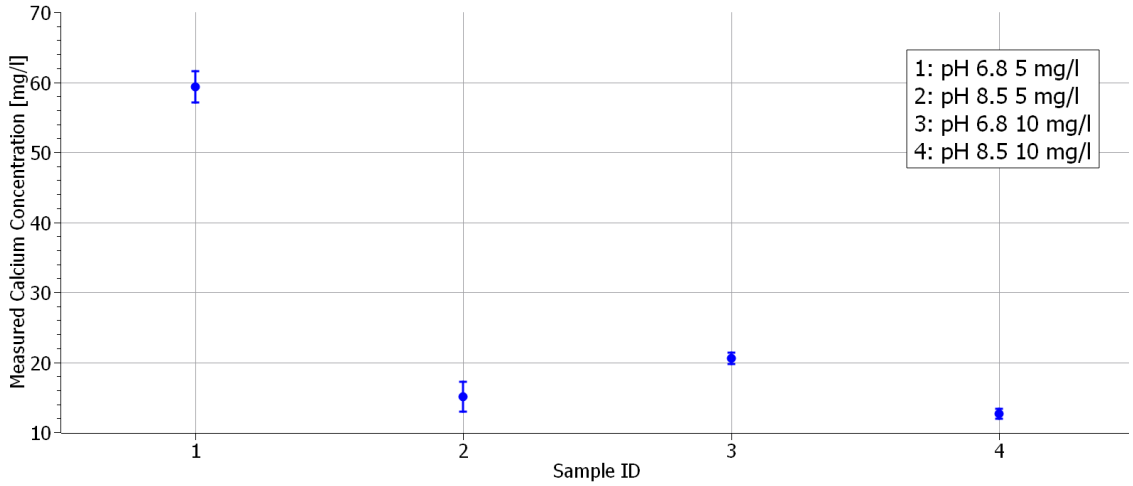


Figure 11: The measured free calcium in dialysis samples from the 2^2-factorial trial. The total calcium concentration at equilibrium was 100 mg/l. Calcium and protein was mixed in the dialysis units for 72h before analyzing samples with the calcium assay.

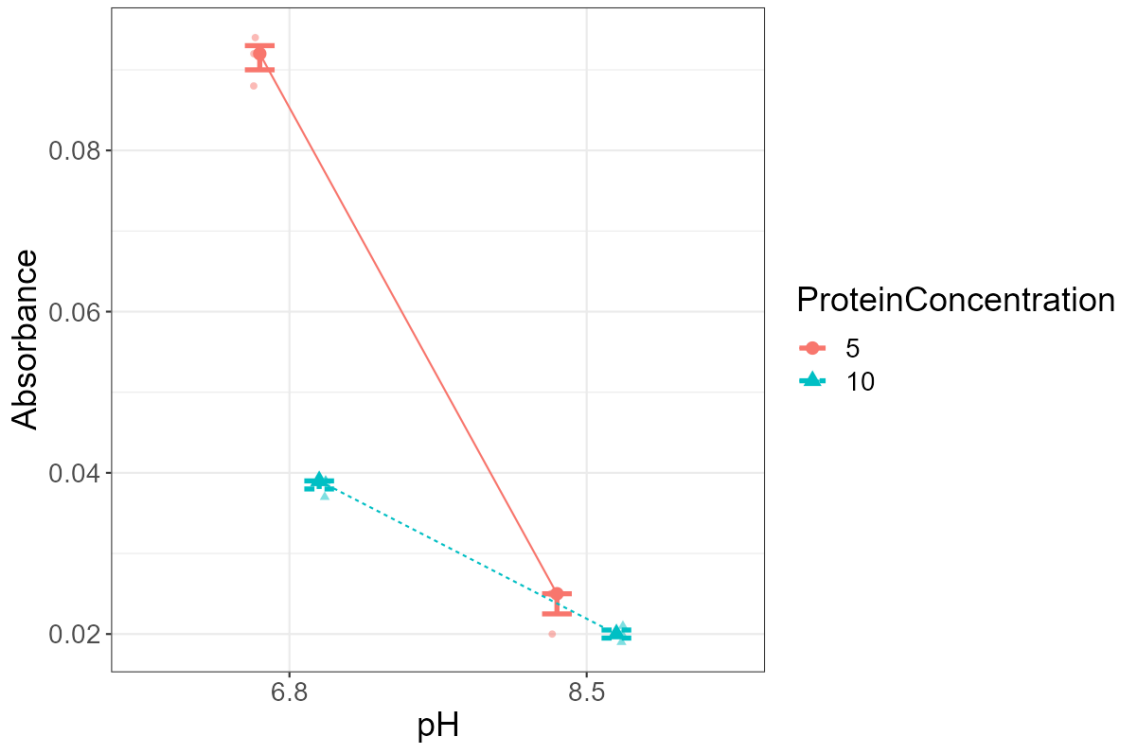


Figure 12: A flexplot of the measured OD of the dialysis samples from the 2^2-factorial trial. The protein concentration was in mg/ml. Calcium and protein was mixed in the dialysis units for 72h before analyzing samples with calcium assay.

The results from the ANOVA on the GLM are presented in table 6. The p-values of the model and the factors are <0.05 which means they are statistically significant. The F-value is the ratio between variation between the groups and within the groups. The factor pH has a greater effect on the response, OD, than protein concentration since the F-value of the pH is greater than the F-value for protein concentration.

Table 6: ANOVA of GLM based on the 2^2-factorial trial. SS is the sum of squares, df is the degrees of freedom, F is the F-statistics, p the statistical probability and η^2 is the effects size.

	SS	df	F	p	$\eta^2 p$
Model	0.00797	2	19.0	<0.001	0.808
pH	0.00559	1	26.6	<0.001	0.747
Protein Concentration	0.00238	1	11.3	0.008	0.557
Residuals	0.00189	9			
Total	0.00986	11			

Equilibrium dialysis was done with a range of calcium concentrations mixed with 5mg/ml SPP in 10 mM Tris-HCl and the results are shown in figure 13. The graph shows a decrease in free calcium concentration compared to the total calcium concentration in each sample.

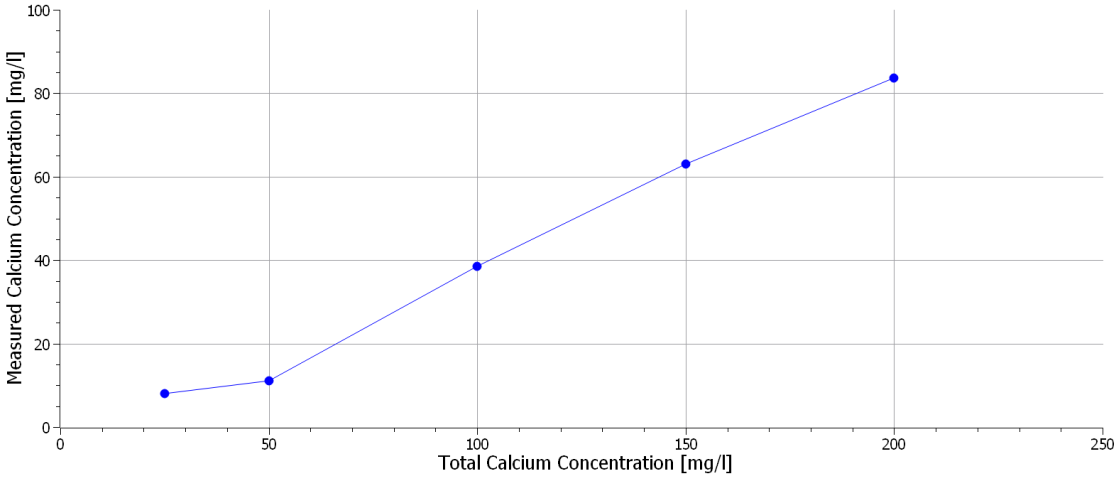


Figure 13: The measured free calcium in dialysis samples with 5 mg/ml SPP in 10 mM Tris-HCl. Calcium and protein was mixed in the dialysis units for 72h before analyzing samples with calcium assay.

4.2.3 Kinetic Calcium Assay

The Kinetic Calcium Assay was done to assess the influence of OCPC on the calcium-protein binding. The results from calcium assay after 0h and 24h incubation time with protein and calcium are presented in figure 14. The total calcium concentration in the samples were 250 mg/l which is one of the standards used in the assay. The OD remains relatively stable in the standards which means the OD of the OCPC-calcium complex is nearly constant. The OD of the protein samples change over time and is more similar to the OD of the total calcium concentration, 250 mg/l, at the end.

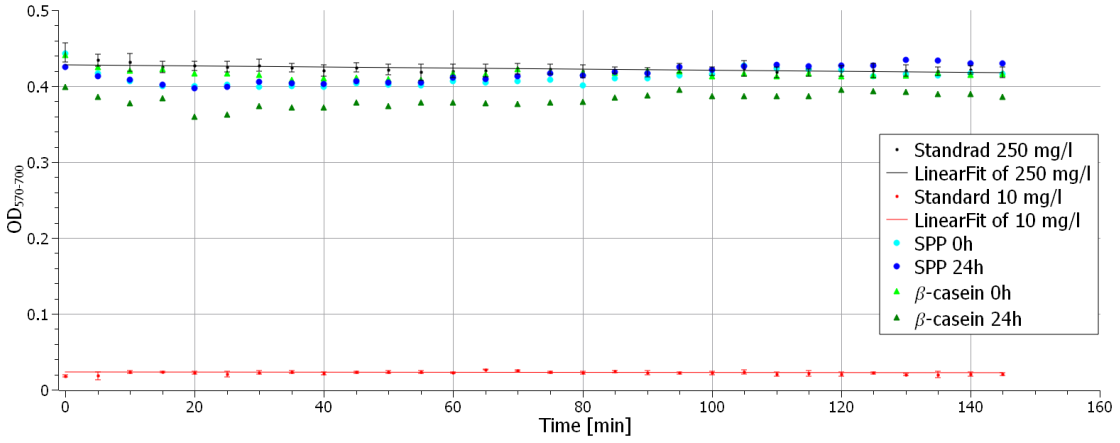


Figure 14: The measured free calcium in samples with 1 mg/ml protein in 10 mM Tris-HCl. Calcium and protein was incubated for either 0h or 24h before analyzing samples with Kinetic Calcium Assay. The total calcium concentration in the samples were 250 mg/l.

4.3 Hydrolysis

4.3.1 SDS-PAGE & HPLC-SEC of hydrolysates

Using HPLC-SEC the high molecular weight peak as well as the first peak around 13.5 min of the SPP disappeared in the hydrolysates, but no clear change in molecular weight distribution or disappearance of peaks could be seen after 14 minutes (fig. 15). However SDS-PAGE results showed clear differences between SPP and hydrolysates with only a band around 5-10 kDa in the hydrolysates and no band around 20 kDa (PI) or 40 kDa (patatin) (fig. 16).

When comparing to standards Ferritin 450 kDa, bovine serum albumin (BSA) 66 kDa, Ovalbumin 45 kDa and Cytochrome C 12 kDa except for the high molecular weight fraction in SPP, that is not retained in the column, all peaks elute below 45 kDa (Appendix E.5). A correlation between retention time and molecular weight was found by taking the 10-log of the molecular weight and making a linear regression. It showed that the three main peaks in the hydrolysates were around 18, 15 and 9 kDa (Appendix E.5). SPP also has peaks at 18, 15 and 9 kDa, these are likely protease inhibitors. There is one peak in SPP around 13.5 min that disappears in the hydrolysates. According to the linear regression it corresponds to 41 kDa, so it is likely patatin (40-45 kDa).

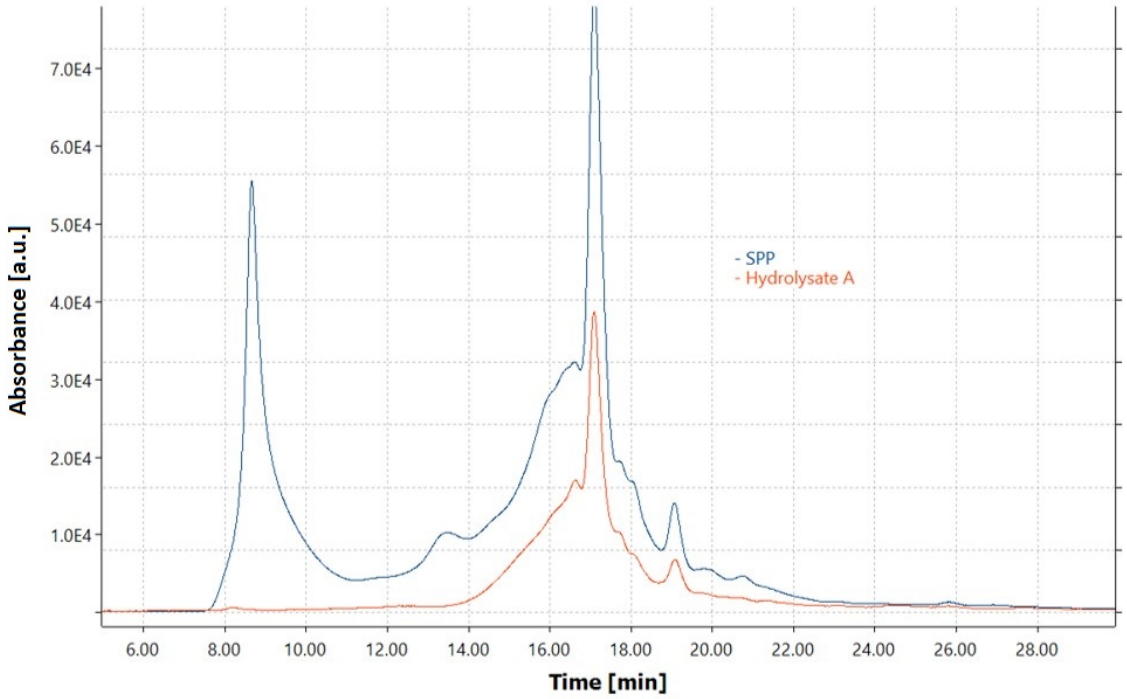


Figure 15: Absorbance at 280 nm for SPP and one of the hydrolysates (A) when running them in HPLC-SEC.

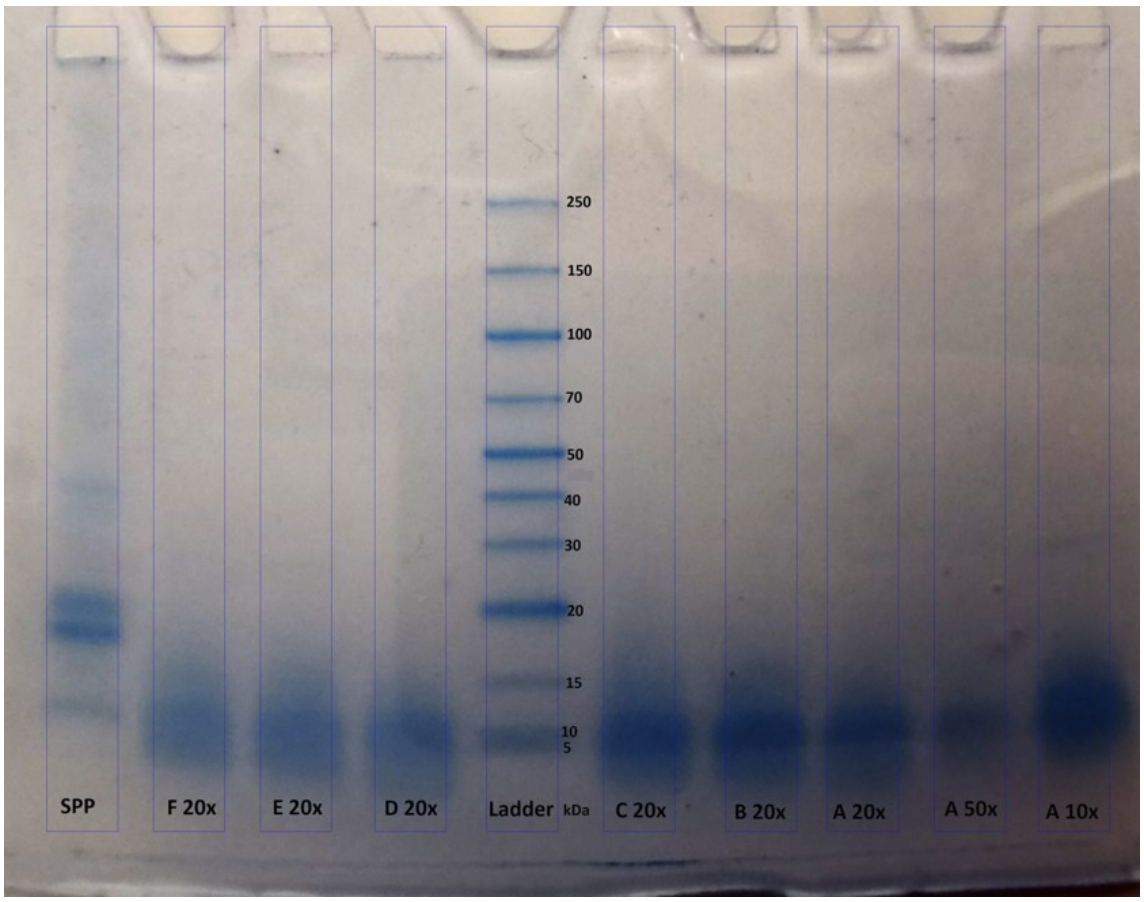


Figure 16: SDS-PAGE gel results for the hydrolysates A-F (see table 2 for a description) diluted 20x, also A with a lower dilution (10x) and a higher dilution (50x) to see if another dilution would be more suitable. SPP in lane 1 as a control to compare the hydrolysates with. A broad-range ladder in lane 5, low molecular weight bands 5 and 10 kDa are not resolved from each other.

4.3.2 pH titration of hydrolysates

Hydrolysates all had similar pH titration behaviour. They all have the largest pH increase per added titrant around pH 5 and pH 8.5 (fig. 17). RALS measurements showed a peak around pH 3.5. Interestingly the peak height generally decreases when the E:S ratio increased, in other words a higher amount of enzyme added seems to lead to a lower peak height (fig. 18).

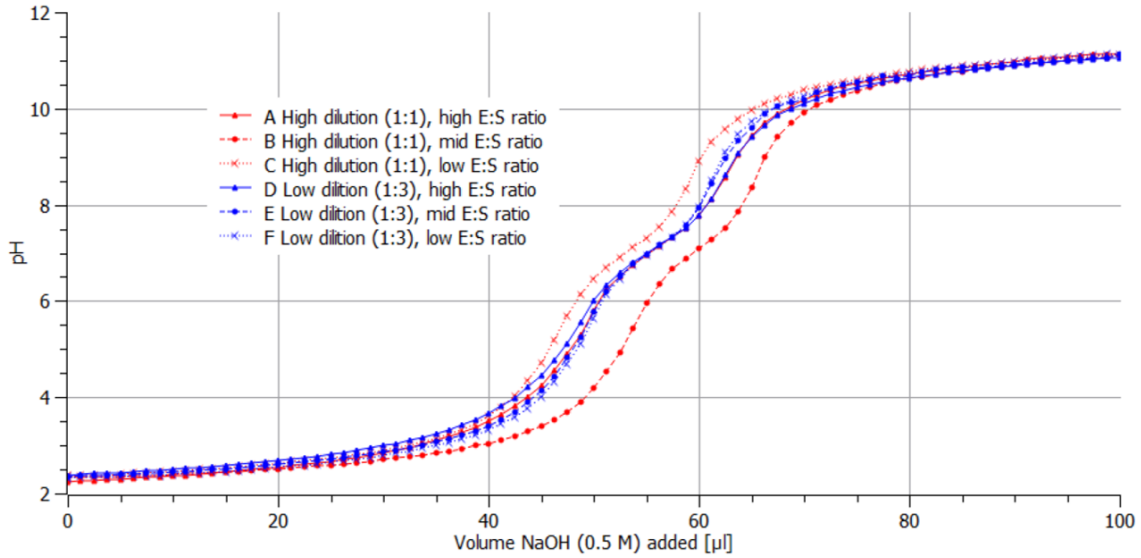


Figure 17: Titration curves for hydrolysates A-F. There is a description of the hydrolysates in table 2.

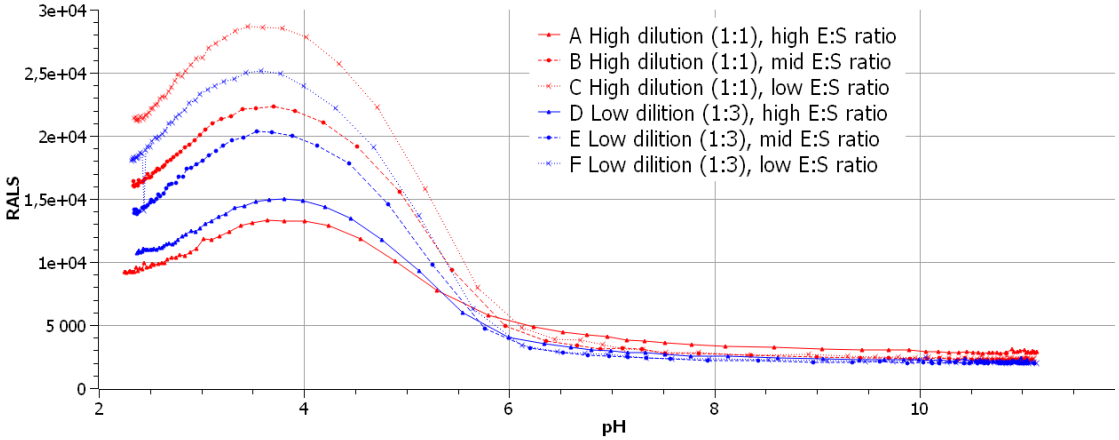


Figure 18: Right-angle light scattering (RALS) variation over pH, measured during pH titration. There is a description of the hydrolysates in table 2.

4.3.3 Calcium titration of hydrolysates

During the calcium titration all hydrolysates behaved similarly: RALS increased rapidly in the beginning and then reached a maximum value. The samples with the highest amount of added calcium (A,D) have a later increase in RALS and reaches a slightly lower maximal value (fig.19).

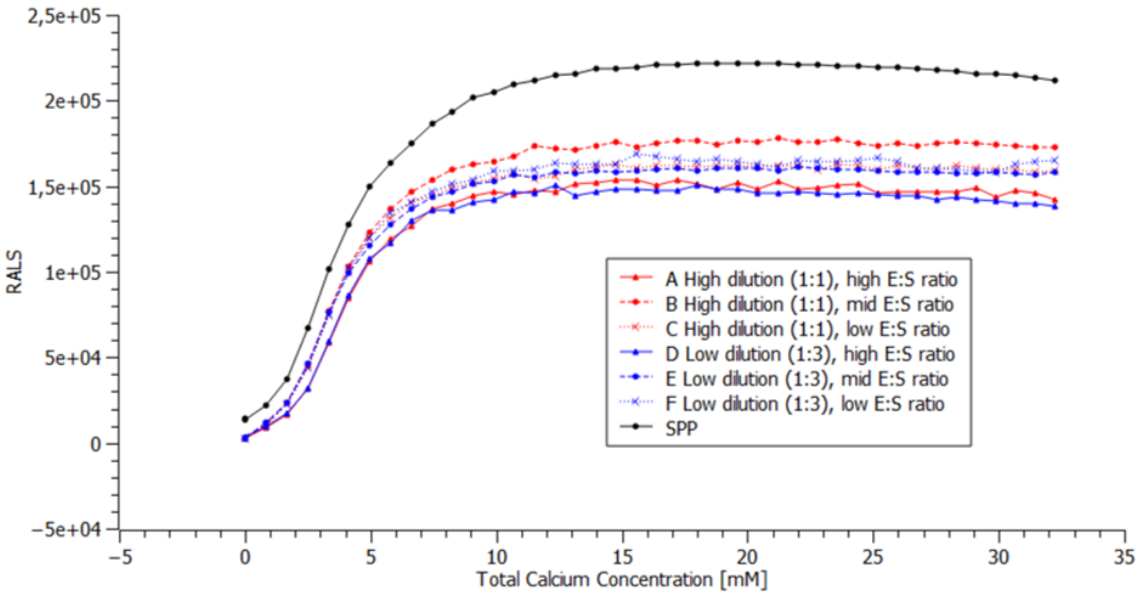


Figure 19: Right-angle light scattering (RALS) variation when titrating calcium into hydrolysates and SPP.

The relative RALS was calculated using the maximum intensity, and the Hill- Langmuir equation was fitted to the data to calculate the dissociation constant, K_d, and the Hill coefficient, n, presented in table 7. The K_d value seems to increase with E:S ratio and is highest at the high dilution factor.

Table 7: Binding constants calculated from Hill-Langmuir equation fitted to the relative RALS data of hydrolysates A-F. K_d is the dissociation constant and n is the Hill coefficient.

Sample ID	E:S ratio	Dilution factor	K_d	n
A	1:8 (High)	2x (High)	49.0 ± 10.1	2.89 ± 0.132
B	1:38 (Mid)	2x	28.8 ± 2.31	2.59 ± 0.0514
C	1:75 (Low)	2x	28.3 ± 2.51	2.70 ± 0.0599
D	1:10 (High)	1.33x (Low)	45.6 ± 11.0	2.89 ± 0.157
E	1:50 (Mid)	1.33x	25.1 ± 2.32	2.60 ± 0.0615
F	1:100 (Low)	1.33x	24.2 ± 3.51	2.51 ± 0.0935

4.4 Calcium Titration with SPP and β-casein

The calcium titration with 2.5 µl injection volume and 60s equilibration time was done with SPP and β-casein and the results of RALS are shown in figure 20 and 21, respectively. The RALS result of the control without protein showed only noise and is presented in figure 29 in Appendix E. In the SPP sample in figure 20, the intensity of RALS increases exponentially as total calcium concentration is added. The maximum intensity is around 15 mg/l. The increase in RALS in figure 21 with β-casein is more linear and smaller in relation to the SPP.

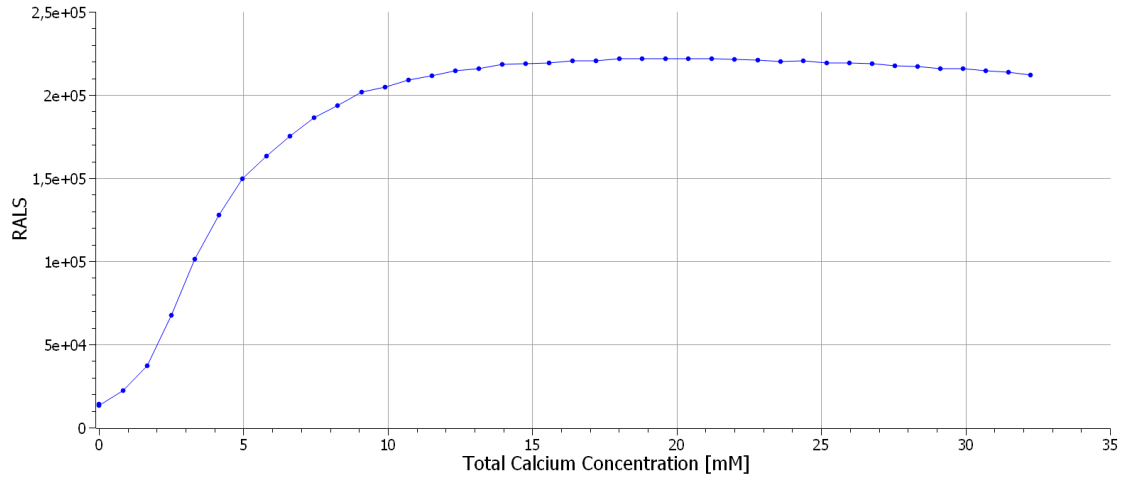


Figure 20: Right-angle light scattering of calcium titration in 1mg/ml SPP in 10 mM Tris-HCl, pH 8.5. The injection volume was 2.5 µl $CaCl_2$ solution with 60s equilibration time over 43 min.

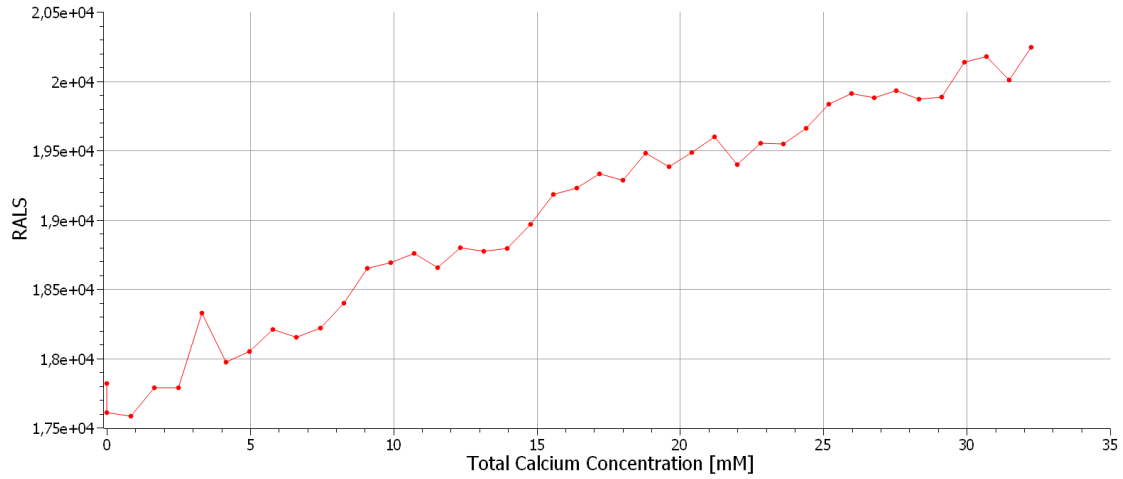


Figure 21: Right-angle sight scattering of calcium titration in 1mg/ml β-casein in 10 mM Tris-HCl, pH 8.5. The injection volume was 2.5 µl $CaCl_2$ solution with 60s equilibration time over 43 min.

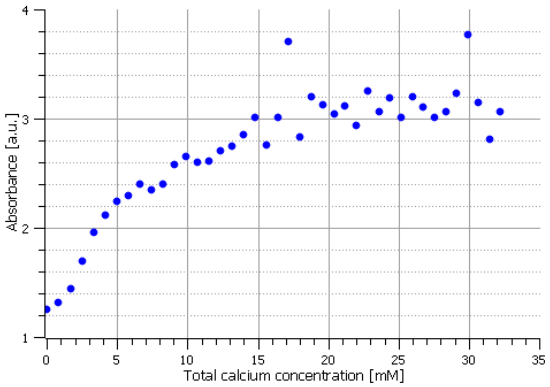
The relative RALS was calculated using the maximum intensity, and the Hill- Langmuir equation was fitted to the data to calculate the dissociation constant, K_d, and the Hill coefficient, n, presented in table 8.

Table 8: Binding constants calculated from Hill-Langmuir equation fitted to the relative RALS data of SPP. K_d is the dissociation constant and n is the Hill coefficient.

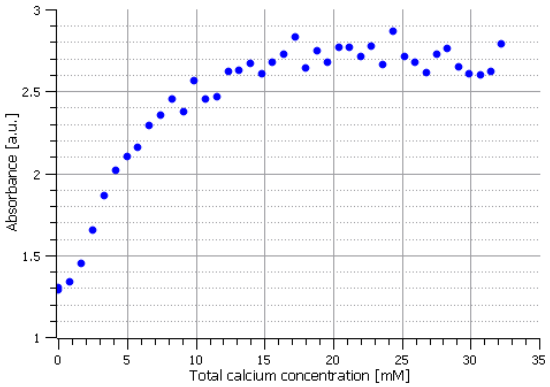
Constants	
K_d	19.4 ± 2.56
n	2.33 ± 0.0826

The absorbance of the SPP and β-casein samples in wavelengths 254 nm and 280 nm are shown in figure 22. The absorbance at both 254 nm and 280 nm, in figure 22a and figure 22b,increase as the total calcium concentration increase in the SPP sample. There is a

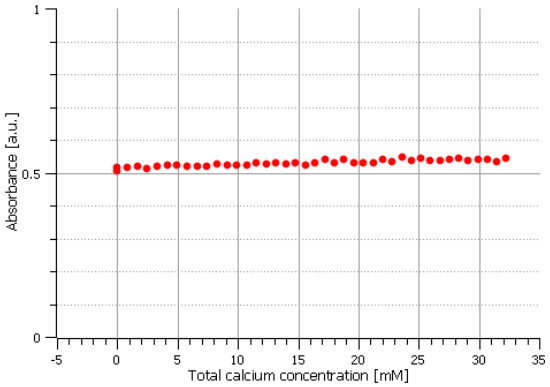
marginal increase in absorbance in the β-casein sample at 254 nm as seen in figure 22c and the absorbance at 280 nm is constant as seen in figure 22d. The change in absorbance at these wavelengths could mean structural transitions as it is the aromatic residues, Tryptophan and Tyrosine, that absorb at 280 nm. The absorbance of these residues are sensitive to changes in the molecular environment and would change in case of structural transitions. As the concentration of SPP is relatively constant, an increase in absorbance at 280 nm would mean the aromatic residues are in more hydrophobic environment.



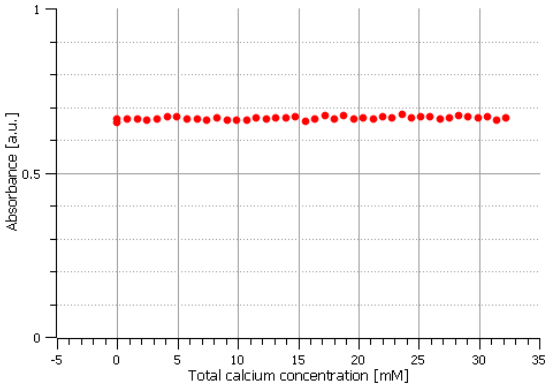
(a) Absorbance of SPP at 254 nm



(b) Absorbance of SPP at 280 nm



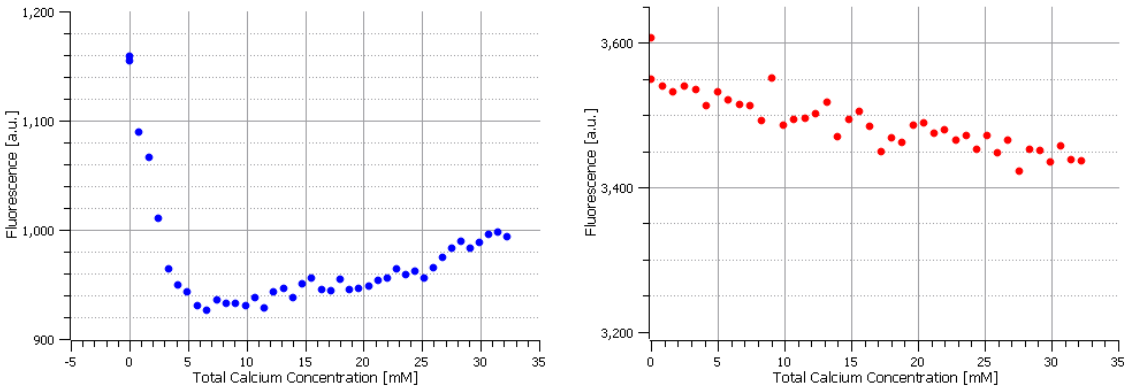
(c) Absorbance of β-casein at 254 nm



(d) Absorbance of β-casein at 280 nm

Figure 22: Absorbance of calcium titration in 1mg/ml protein in 10 mM Tris-HCl, pH 8.5. The injection volume was 2.5 µl $CaCl_2$ solution with 60s equilibration time over 43 min.

The fluorescence of the SPP and β-casein samples measured during the calcium titration is shown in figure 23a and figure 23b respectively. The excitation was at 280 nm and the emission maximum was at about 350 nm. The emission maximum shifted to 345 nm when calcium was added to SPP. In β-casein, the emission maximum was at 352 nm. The excitation at 280 nm and emission maximum around 350 nm measures the fluorescence of fluorophores in Tryptophan and Tyrosine residues. The emission wavelength tends to decrease as the residues are buried in a more hydrophobic environment. The decrease in fluorescence intensity could be due to protein-protein interaction or calcium interaction with the fluorophores in the protein as this could quench the fluorescence. The change in intensity and shift in emission wavelength could be due to conformational changes induced by factors like calcium binding or protein self-assembly, or it could be due to alterations in the solvation environment around the protein.



(a) Fluorescence of SPP at 345 nm

(b) Fluorescence of β-casein at 352 nm

Figure 23: Fluorescence of calcium titration in 1mg/ml protein in 10 mM Tris-HCl, pH 8.5. The injection volume was 2.5 µl $CaCl_2$ solution with 60s equilibration time over 43 min.

5 Discussion

5.1 Characterization of SPP

As expected the SDS-PAGE results show that SPP contain a protein at 40 kDa that is likely patatin and and two at around 20 kDa which are likely protease inhibitors. There are also some bands around 10 kDa which are probably also protease inhibitors. There seems to be some protein at 100 kDa that mostly end up in the pellet, these could be high molecular weight proteins. These results are also supported by the results from HPLC-SEC that shows that SPP contain a compund with molecular weight 41 kDa and some more compunds around 10-20 kDa. Overall the SDS-PAGE result indicates that both soluble and insoluble fraction of SPP contains both patatin and protease inhibitors, as expected. The bands corresponding to protease inhibitors are thicker and darker in all samples indicating that there is a larger fraction of protease inhibitors, which is not expected since it should be roughly the same amount of protease inhibitors and patatin in potato protein isolate. It is consistent with the peaks in HPLC-SEC being higher around times corresponding to 15-18 kDa than 41 kDa. It might be affected by the (to us unknown) extraction method used to avoid coagulation and denaturation of the proteins and maintain a high solubility, which might favour extraction of the protease inhibitors.

Results of the dry matter analysis shows as expected that SPP has a considerably higher solubilty than FPP. The solubility of SPP dropping around pH 5 is reasonable since it is close to the isoelectric point of the patatin where it could form aggregates and lose solubility. This is also supported by the pH titration peak in RALS around pH 5. There was however no reduction of solubility or increase in RALS around pH 8.5 which is the isoelectric point of the protease inhibitors. This indicates that even at their isoelectric point the protease inhibitors does not form larger aggregates and become insoluble.

5.2 Calcium Assay

From the figures 7 and 8 we could see that the OD of OCPC-calcium complex was linearly proportional to the calcium concentration and the standard curves show a high goodness of fit overall. At low concentrations 1-5 mg/l, the resolution is poor and not linear. The size of the error was adequate in the calcium concentration ranges 10-250 mg/l, since the purpose

was only to get an approximate of the free and bound calcium. The observation that OD shifted when calcium was dissolved in different solutions could be due to the different pH and hydration of the calcium. The effect was not thoroughly tested but merely an observation that was made during the experiments to establish a method for calcium assay. The assay's sensitivity to temperature was also an observation that was made when the calcium assay was done using room temperature OCPC and cool OCPC. The limitations of the calcium assay with OCPC makes it difficult to perform a reliable analysis since there are many opportunities for error but if performed with experience and in a well-controlled environment the reliability of the results was adequate for the purpose of this project.

Another limitation with the calcium assay with OCPC was the issue of measuring the calcium directly from a mix of protein and calcium. The validation of calcium assay with β-casein by directly sampling from the protein-calcium mix did not show any clear sign of calcium binding as the free calcium that was measured was about the same as the total calcium concentration. One explanation could be that the protein mix could contain magnesium ions which have shown to interfere with OCPC calcium assays but as a control of only protein solution with no calcium was used, it seemed unlikely. The validation with β-casein, when sampling directly from the mix of protein and calcium, might have failed due to insufficient time for the β-casein-calcium binding. Subsequent experiments showed that β-casein requires longer time to form micelles and bind to calcium. For instance, in Kinetic Calcium Assay, the calcium assay done after 24h incubation of β-casein and calcium did show a lower free calcium concentration.

The same problem with directly sampling the calcium for the calcium assay from the SPP-calcium mix was observed. The reason could be insufficient incubation time with protein and calcium but it could also be due to a high dissociation constant. When OCPC is added to the calcium sample, the formation of OCPC-calcium complexes starts which decreases the concentration free calcium which affects the equilibrium for the binding of SPP-calcium complex. This in turn could lead to dissociation of calcium and protein. The calcium assay would then show a higher concentration free calcium when measuring than it was initially in the protein-calcium solution. The Kinetic Calcium Assay after 24h incubation in figure 14 supports this hypothesis. The OD of the sample with SPP increases over time until it is adjoining the calcium standard which is the total calcium concentration in the sample. It indicates that calcium and SPP dissociate as the free calcium increases over time. Using our method for calcium assay, with relatively long preparation time and incubation time, this phenomena was an issue.

To solve this issue, equilibrium dialysis was used so the protein-calcium reaction could be performed in one chamber while sampling was done in another without protein. The calcium concentration which would be divided evenly between chambers, could be measured without the issue of dissociation due to OCPC-calcium complex formation. The procedure of these experiments proved to be ineffective in regards to time and prone to error. The measurements with β-casein did show a decrease in free calcium but also an unexpected variation in the results. The deviations might have been caused by leakage or spillage during the preparation of samples in dialysis units. The dialysis could also have been affected by foaming which the proteins samples were prone to. The dialysis with SPP showed that the measured free calcium concentration was lower than the total calcium concentration in the different samples which indicated that some sort of SPP-calcium binding occurred. A more substantial experiment with statistical analysis would have to be done to confirm.

The GLM and ANOVA of the 2^2-factorial trial showed that pH and protein concentration were significant factors. The measured free calcium concentration was similar for 5 mg/ml and 10 mg/ml SPP at pH 8.5 but very different at pH 6.8 as seen in figure 11. Both

pH 6.8 and pH 8.5 are above one of the pIs of SPP which means the proteins would have a negative net charge and be more likely to bind to calcium as explained in Theory. As seen in the pH titration and dry matter analysis, the solubility of SPP is higher at pH 8.5. This could explain the lower free calcium concentration in samples at pH 8.5 compared to pH 6.8 since it would be favourable for SPP-calcium binding if the proteins are more soluble and have a negative net charge. The flexplot in figure 12 of the 2^2-factorial trial also shows that the effect of SPP concentration, on the measured absorbance, is bigger at the lower pH compared to higher. The results from this trial could indicate that the solubility and state of the proteins are very influential in promoting SPP-calcium binding.

5.3 Hydrolysis

The initial HPLC-SEC results of hydrolysate and SPP stock looked similar, indicating that no or very little hydrolysis had occurred. With the hypothesis that protease inhibitors were inhibiting hydrolysis a heat treatment was added based on literature [8]. HPLC-SEC once again revealed little changes indicating low degree of hydrolysis. However SDS-PAGE results showed large changes with only a very faint band around 10 kDa in the hydrolysates. This is an indication that hydrolysis occurred, but could also be due to too high dilution in combination with heat treatment, hydrolysis and centrifugation that lowers the concentration of protein in the samples. When heat treating the SPP a gel is formed so it needs to be diluted to be able to mix well with the Alcalase and have a homogenous mixture. When a new gel electrophoresis was done with lower dilutions clearer bands around 5-10 kDa appeared, indicating that hydrolysis was successful since it is the most probable explanation. A reason for the contradictory HPLC-SEC and SDS-PAGE results could be that there are intramolecular forces such as hydrophobic interactions within the proteins, so even after hydrolysis the peptides could stay together as larger units. In SDS-PAGE the sample pre-treatment involves solubilizing hydrophobic parts with the detergent SDS, which would make the protein compex divide into peptides. The samples are not pre-treated with detergent before HPLC-SEC, it was considered but not attempted.

All hydrolysates have a similar change in pH with added titrant, with pH 5 and 8.5 being where the pH changes the most. Measurements of RALS indicates some aggregates forming with a maximum around pH 3.5, a lower pH compared to SPP that has a maximum around ca pH 5. The peaks are also lower, $< 30\,000$ for hydrolysates with an estimated ca 1 mg/ml protein (rough estimation) compared to $>150\,000$ with ca 1 mg/ml SPP. There seems to be a correlation that higher amount of enzyme leads to a lower peak, which is an indication of a higher degree of hydrolysis.

The hydrolysate samples all react with calcium and form aggregates at a size that affect RALS. They reach a saturation after some addition of calcium as seen by the RALS reaching a maximal value and then staying relatively constant over time as more calcium is added.

5.4 Calcium Titration with SPP

Titration of $CaCl_2$ solution in samples with SPP resulted in and exponential growth in RALS intensity as seen in figure 20. The increase in RALS means there is a formation of larger protein assemblies in the sample. As it increases with calcium titration it indicates the calcium influences the aggregation of protein. The absorbance at 254 nm and 280 nm exponentially increased and the fluorescence at emission maximum exponentially decreased as calcium was added to SPP. As the absorbance and fluorescence at these wavelengths occur due to contributions from the residues Tryptophan and Ty-

rosine, these changes can be connected to conformational changes due to protein-ligand interactions. The absorbance measurements show that aromatic residues are exposed to a more hydrophobic environment which could mean they are involved in protein-protein or protein-ligand interactions. By connecting the results from these measurements and the indication of calcium binding in the dialysis and calcium assay experiments, it is probable that these changes are due to SPP-calcium binding. The Hill-Langmuir equation was fitted to the relative RALS of SPP and SPP hydrolysate, as the relative RALS was assumed to be the fraction of occupied receptor by the ligand, in this case fraction of SPP occupied by calcium. The K_D of SPP and SPP hydrolysates were quite high which means dissociation of SPP and calcium is high. Dissociation of SPP-calcium complex is good when the calcium needs to be released for absorption in the body but it might be too high to hold calcium throughout the digestive system until the small intestine.

The Hill coefficients of SPP and SPP hydrolysates show positive cooperativity binding. This means the binding of one ligand facilitates binding of subsequent ligands at other sites on the complex.

6 Conclusion

To conclude the SPP sample contained soluble patatin and protease inhibitors. Solubility was around 54 % at pH 8.5, decreased around pH 5 and was high at both acidic and alkaline pH. The calcium assay works for approximate determination of calcium concentration in pure samples when temperature, incubation time and pH is well controlled. To be able to measure free calcium in impure samples equilibrium dialysis could be a solution but the setup used in this study would need to be improved on efficiency and robustness. The results from the dialysis experiments indicated that protein-calcium binding occurred but due to the uncertainty of the method, the validity of the results are unsure and we can not conclude with certainty if SPP is capable of forming a complex with calcium. However, with the results from calcium titration experiments together with indication of calcium binding in the dialysis and calcium assay, it is highly likely that there is SPP-calcium binding.

Fitting the Hill-Langmuir model to the calcium titration gave K_d 19.4 mM for SPP, and between 24-49 mM for hydrolysates. Binding with $K_d > 1$ mM is considered to have low affinity [23], and the values generated from the Hill-Langmuir model are considerably greater than that. According to the Hill-Langmuir model, SPP has low affinity for calcium and the SPP-calcium complex is prone to dissociation, especially the hydrolysates.

Using Alcalse worked for hydrolysing SPP, but it seems like the peptides mostly stay together in the protein structures, possibly due to hydrophobic interactions. They did however behave differently compared to SPP during the calcium titration and had a higher K_d compared to SPP which indicates some changes in functionality towards a lower calcium affinity. More research is needed to better understand how the hydrolysates bind to calcium, it would be interesting to run a kinetic calcium assay with the hydrolysates but this was not done due to lack of time.

7 Contributions

The initial idea and plan of this thesis was done by Prof. Dr. Cedric Dicko and Prof. Dr. Lars Nilsson and they have been helpful along the way with input on how to proceed. Prof. Dr. Cedric Dicko has helped us with instructions in the lab and with running some of the instruments we have used. Deep Bhattacharya ran most of the SDS-PAGE gels and helped with instructions on how to do it. Camille Bancel and Justine Bercq helped do all the lab work for the dry matter analysis of SPP and FPP with instructions from us. We, Signe and Erika, have done the other work for this thesis including planning, conducting experiments in the lab, data analysis, and writing. We both contributed equally in the lab and with the writing of this thesis. The potato protein samples were a gift from the Swedish company Lyckeby.

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First of all, we want to thank our supervisor Prof. Dr. Cedric Dicko for letting us do this project in your group and for providing insights, moral support, optimistic encouragement and helping us throughout the project. We also want to thank Prof. Dr. Lars Nilsson for also supervising our project and providing valuable insights and guidance. We are grateful to you for the opportunity to do this project and lending us support to explore our ideas. We also want to thank our fellow master thesis student, Deep Bhattacharya, for helping us with SDS-PAGE, and Camille Bancel and Justine Bercq for assisting us with the dry matter analysis. We are also grateful for Dr. Pamela Soto Garcia for lending us a hand in the lab and her reassuring optimism. Addtionally, we want to thank Lyckeby for their cooperation and providing us with potato protein samples. Last but not least, a thank you to our examiner, Prof. Dr. Lieselotte Cloetens, for taking the time to read and evaluate our thesis.

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Method on how to purify PFJ to get patatin.

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9 Appendix

A Protocols for machines

A.1 Calcium Assay protocol

Absorbance: Absorbance values are pathlength corrected based on waterpeak. Basic settings: Measurement type: Absorbance. Microplate name: NUNC 96. Endpoint settings. No. of flashes per well: 20. Scan mode: None. Optic settings: No. Excitation 1. 570 nm 2. 700 nm. General settings: Settling time [s]:0.1. Reading direction:bidirectional, horizontal left to right, top to bottom. Target temperature [°C]:set off.

A.2 Kinetic Calcium Assay protocol

Absorbance:Absorbance values are pathlength corrected based on waterpeak. Basic settings: Measurement type: Absorbance. Microplate name: NUNC 96. Kinetic settings: No. of cycles: 30. Cycle time [s]: 300. No. of flashes per well and cycle: 20. Scan mode: spiral averaging. Scan diameter [mm]: 3.

Optic settings: No. Excitation. 1.570 nm. 2.700 nm.

Shaking settings: Shaking 1. Shake:before first cycle. Movement:double orbital. Frequency [rpm]: 300. Time [s]: 30.

General settings: Settling time [s]: 0.1. Reading direction:bidirectional, horizontal left to right, top to bottom. Target temperature [°C]:set off.

A.3 Labbot pH Titration

Procedure SPP pH titration
Meter is: Orion Star A215 (pH).
Initial temperature 20 deg C . Baseline 30 seconds.
Concentration titration to a total volume of 100 ul in 2.5 ul steps.
Normal loading.
Mixing time 0 seconds. Equilibration time 30 seconds.
Continous stirring at speed 6.
Titrant is NaOH at 500 mM.
Measure RALS and record spectra using the following 1 detectors:
Detector 1 records the range 250 - 770 nm with 4 averages.
Mode: absorbance
The estimated run time is 23 minutes.
protein concentration: 1mg/ml

A.4 Labbot Calcium Titration

Procedure SPP titration calcium
Meter is: Orion Star A215 (pH).
Initial temperature 20 deg C . Baseline 30 seconds.
Concentration titration to a total volume of 100 ul in 2.5 ul steps.
Normal loading.
Mixing time 0 seconds. Equilibration time 60 seconds.
Continous stirring at speed 6.
Titrant is Calcium at 1000 mM.
Measure RALS and record spectra using the following 2 detectors:
Detector 1 records the range 225 - 600 nm with 4 averages.
Mode: absorbance
Detector 2 records the range 285 - 600 nm with 4 averages.
Mode: fluorescence. ExLight : 280:10. IntTime: 220 ms.
The estimated run time is 43 minutes.
protein concentration: 1mg/ml

B Hydrolysis experimental design

Table 11: Experimental design of the hydrolysis.

Sample ID	E:S ratio [mg:mg]	Dilution factor	SPP stock [μl]	Milli-Q water [μl]	10x Alcalase [μl]	Total volume [μl]
A	1:8	1x	750	700	50	1500
B	1:38	1x	750	740	10	1500
C	1:75	1x	750	745	5	1500
D	1:10	1.33x	1000	283	50	1333
E	1:50	1.33x	1000	323	10	1333
F	1:100	1.33x	1000	328	5	1333

C Calcium Assay Calculations

The OD of the OCPC-calcium complex is corrected according to equation 7.

$$OD_{correct} = (OD_{sample,570} - OD_{blank,570}) - (OD_{sample,700} - OD_{blank,700}) \quad (7)$$

The linear relation between calcium concentration and OD is calulated using linear regression by fitting equation 8 to the data.

$$y = a \cdot x + b \quad (8)$$

The y is $OD_{correct}$ and x is the calcium concentration. Equation 8 is rewritten to equation 9 and used to calculate the calcium concentration.

$$x = \frac{(y - b)}{a} \quad (9)$$

D Equilibrium Dialysis Setup

SpinDialyzer^{TM}-units (Harvard Apparatus) were used for equilibrium dialysis. The SpinDialyzer^{TM} was not use as intended, as they were not spun around, but instead a magnetic stirring bar was used. The SpinDialyzer^{TM} is shown in figure 24.

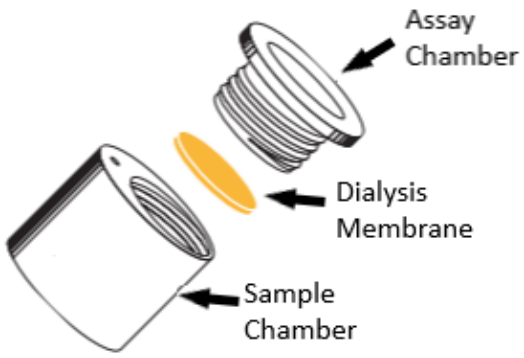

Figure 24: Illustration of the SpinDialyzer^{TM}.

E Supplemental Results

E.1 Dry matter analysis data

ID	Sample	pH	Aluminium foil weight (g)	Alu. Foil + supernatant (g)	Alu. Foil + supernatant after drying (g)	Pellet + eppendorf tube (g)	Dry pellet + eppendorf tube (g)	Clean eppendorf tube (g)	Pellet (g)	Supernatant (g)	Total mass (mg)	Total mass (g)	%Solubility	%Insoluble
1	3.1	3	0.2483	2.1218	0.2587	1.2619	1.1282	1.1186	0.0096	0.0104	20	0.02	52	48
2	3.2	3	0.1769	1.9642	0.1862	1.2559	1.1359	1.1265	0.0094	0.0093	20	0.02	46.5	47
3	3.3	3	0.2328	2.024	0.2417	1.2318	1.1256	1.1163	0.0093	0.0089	20	0.02	44.5	46.5
4	5.1	5	0.2546	2.0844	0.2588	1.2518	1.134	1.1208	0.0132	0.0042	20	0.02	21	66
5	5.2	5	0.3026	1.8993	0.3074	1.2385	1.1358	1.1229	0.0129	0.0048	20	0.02	24	64.5
6	5.3	5	0.2493	1.1166	0.2516	1.2545	1.123	1.1099	0.0131	0.0023	20	0.02	11.5	65.5
7	7.1	7	0.2761	2.1518	0.2872	1.2115	1.1253	1.1183	0.007	0.0111	20	0.02	55.5	35
8	7.2	7	0.2592	2.0595	0.27	1.2334	1.1309	1.1239	0.007	0.0108	20	0.02	54	35
9	7.3	7	0.2625	2.114	0.2735	1.214	1.1205	1.1137	0.0068	0.011	20	0.02	55	34
10	8.1	8	0.1698	2.0134	0.1819	1.2513	1.1236	1.1172	0.0064	0.0121	20	0.02	60.5	32
11	8.2	8	0.2896	1.0978	0.2957	1.245	1.1277	1.1216	0.0061	0.0061	20	0.02	30.5	30.5
12	8.3	8	0.1751	1.9455	0.187	1.2442	1.1323	1.1267	0.0056	0.0119	20	0.02	59.5	28
13	9.1	9	0.3319	2.1888	0.3454	1.2306	1.1112	1.1064	0.0048	0.0135	20	0.02	67.5	24
14	9.2	9	0.2522	1.4428	0.1942	1.2566	1.1153	1.1104	0.0049	-0.058	20	0.02	-290	24.5
15	9.3	9	0.3158	2.1072	0.3272	1.2352	1.1138	1.1085	0.0053	0.0114	20	0.02	57	26.5
16	10.1	10	0.3019	2.1489	0.3163	1.2273	1.1101	1.1057	0.0044	0.0144	20	0.02	72	22
17	10.2	10	0.2792	2.1485	0.2936	1.2507	1.1231	1.1186	0.0045	0.0144	20	0.02	72	22.5
18	10.3	10	0.2376	2.1573	0.2522	1.259	1.1157	1.1114	0.0043	0.0146	20	0.02	73	21.5

I did a mistake when I wrote the weight of the Alu. Foil + supernatant AD

Figure 25: Raw data of weight measurements during dry matter analysis of SPP.

ID	Sample	pH	Aluminium foil weight (g)	Alu. Foil + supernatant (g)	Alu. Foil + supernatant after drying (g)	Pellet + eppendorf tube (g)	Dry pellet + eppendorf tube (g)	Clean eppendorf tube (g)	Pellet	Supernatant	Total mass (mg)	Total mass (g)	% Solubility	% Insolubility
1	3.1	3	0.2511	2.0753	0.2531	1.2002	1.0977	1.0893	0.0084	0.002	20	0.02	10	42
2	3.1	3	0.2477	1.3114	0.2487	1.1909	1.105	1.0923	0.0127	0.001	20	0.02	5	63.5
3	3.3	3	0.2537	2.0862	0.2553	1.2091	1.1136	1.0999	0.0137	0.0016	20	0.02	8	68.5
4	5.1	5	0.2787	2.1371	0.2794	1.2319	1.1172	1.1018	0.0154	0.0007	20	0.02	3.5	77
5	5.2	5	0.2726	2.0836	0.2733	1.1872	1.0998	1.0874	0.0124	0.0007	20	0.02	3.5	62
6	5.3	5	0.2377	1.5204	0.2387	1.2253	1.1132	1.1016	0.0116	0.001	20	0.02	5	58
7	7.1	7	0.2195	1.5052	0.2209	1.2024	1.1064	1.0928	0.0136	0.0014	20	0.02	7	68
8	7.2	7	0.2499	2.0936	0.253	1.1707	1.1013	1.0933	0.008	0.0031	20	0.02	15.5	40
9	7.3	7	0.3186	2.2127	0.3197	1.1539	1.0945	1.0861	0.0084	0.0011	20	0.02	5.5	42
10	8.1	8	0.269	2.1128	0.2702	1.1996	1.0995	1.0856	0.0139	0.0012	20	0.02	6	69.5
11	8.2	8	0.2767	2.1741	0.2778	1.1839	1.0983	1.0871	0.0112	0.0011	20	0.02	5.5	56
12	8.3	8	0.2401	1.7439	0.2414	1.2041	1.107	1.0943	0.0127	0.0013	20	0.02	6.5	63.5
13	9.1	9	0.2331	2.085	0.2344	1.2053	1.0962	1.0855	0.0107	0.0013	20	0.02	6.5	53.5
14	9.2	9	0.2518	1.6076	0.2544	1.2171	1.1008	1.0865	0.0143	0.0026	20	0.02	13	71.5
15	9.3	9	0.2724	2.1858	0.2737	1.2405	1.1134	1.1017	0.0117	0.0013	20	0.02	6.5	58.5
16	10.1	10	0.2439	2.0894	0.2453	1.21	1.1022	1.0921	0.0101	0.0014	20	0.02	7	50.5
17	10.2	10	0.2537	2.1322	0.2553	1.2064	1.098	1.0896	0.0084	0.0016	20	0.02	8	42
18	10.3	10	0.2446	2.1194	0.2462	1.2136	1.1027	1.093	0.0097	0.0016	20	0.02	8	48.5

Figure 26: Raw data of weight measurements during dry matter analysis of FPP.

E.2 Establishing Calcium Assay

Th OD was measured at different times after adding OCPC to assess the effect on incubation time to establish the calcium assay method. The results are presented as linear fits of the data in figure 27. The error bars of the data points also decrease from 5 min to 20 min.

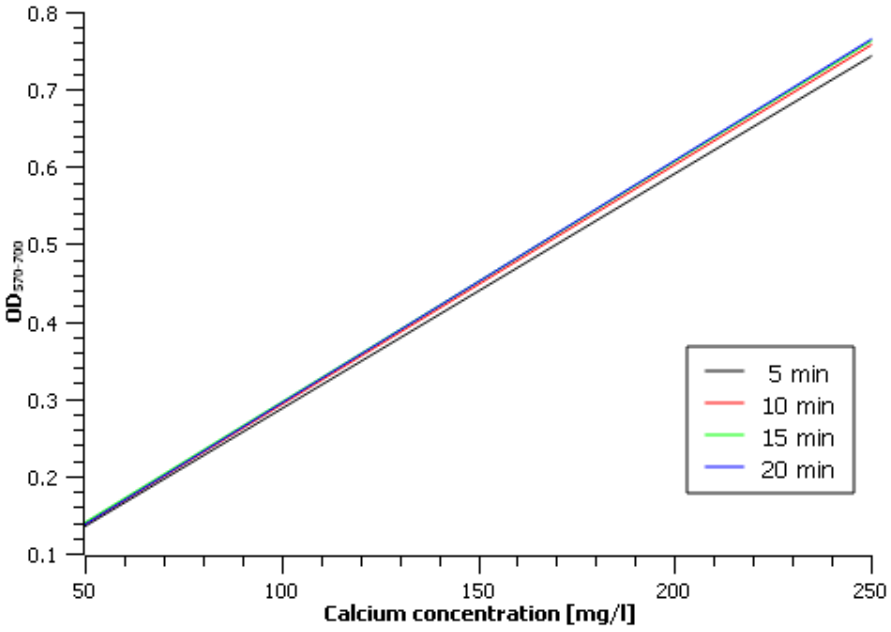

Figure 27: Linear fit of measured OD of calcium standards at different incubation times.

E.3 Establishing Equilibrium Time

To establish the equilibration time for the equilibrium dialysis, calcium standard samples were used to measure and see when the calcium concentration reached 100 mg/l. The result is presented in figure 28.

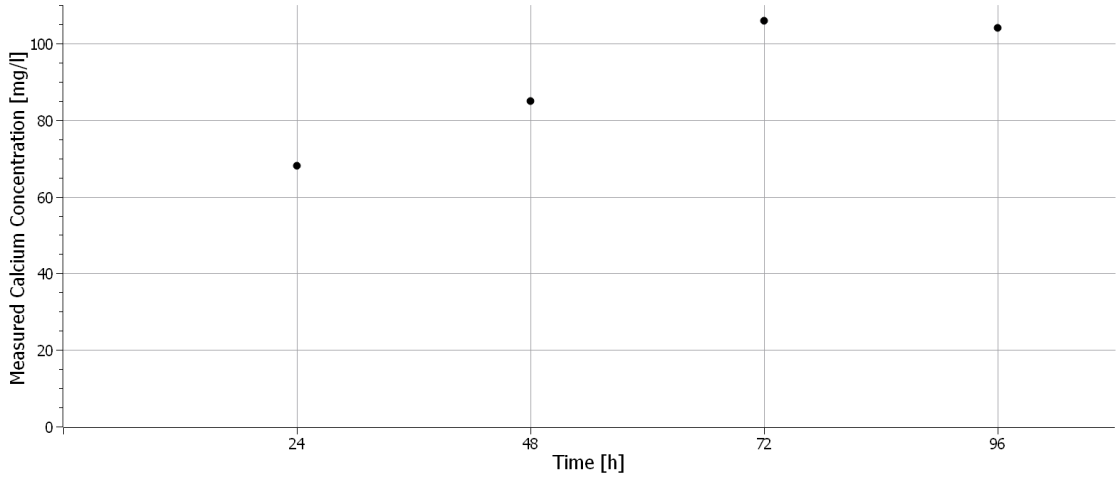

Figure 28: The measured free calcium in dialysis control samples in 10 mM Tris-HCl, with 100 mg/l calcium at equilibrium. The dialysis control samples were measured after 24h, 48h, 72h and 96h with calcium assay.

E.4 Calcium Titration Control

The result of the calcium titration control without protein is presented in figure 29.

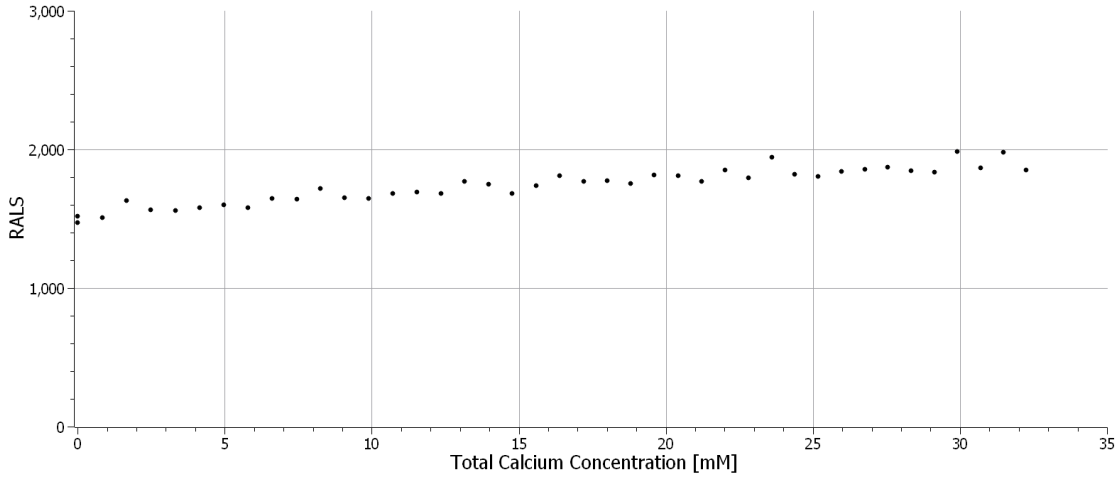

Figure 29: Right-Angle Light scattering of control sample for calcium titration with 10 mM Tris-HCl.

E.5 HPLC-SEC of hydrolysates

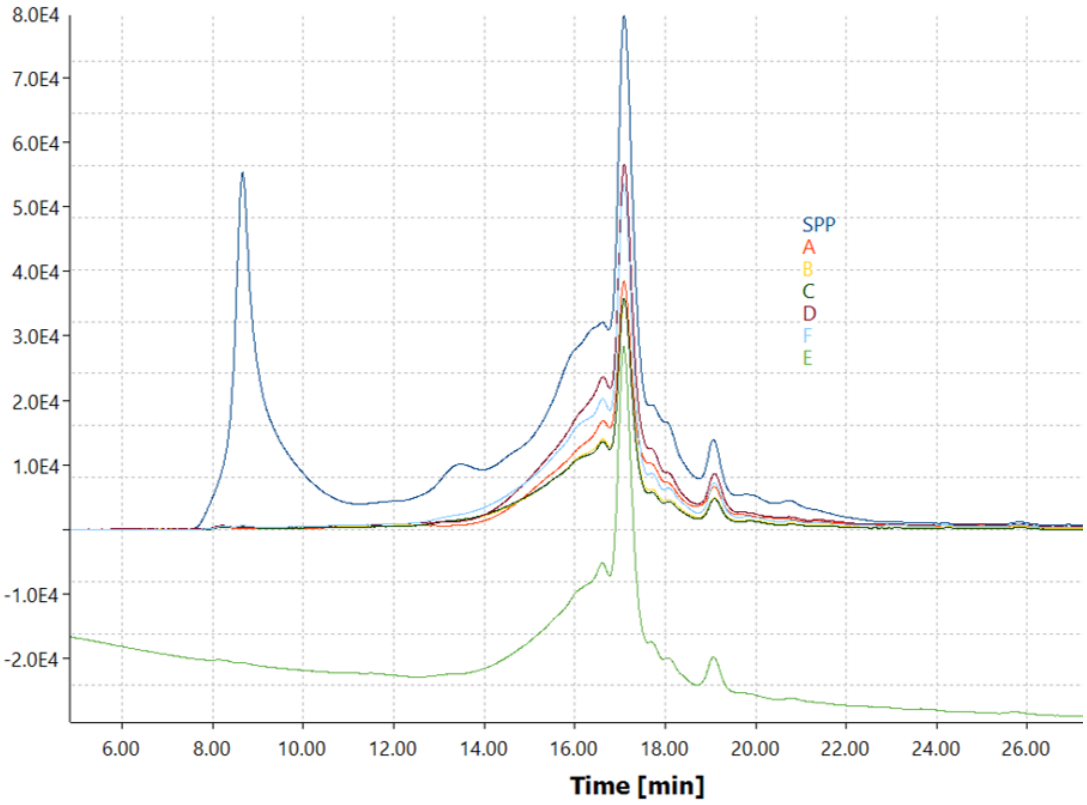

Figure 30: HPLC-SEC results for all 6 hydrolysates A-F and SPP.

Table 12: HPLC-SEC results on elution times for molecular weight standard peaks.

Sample	Molecular weight [kDa]	Time [min]
Aminobenzoic acid	0.14	17.968
Cytochrome C	12	17.98
Ovalbumin	45	12.962
BSA	66	12.035
Ferritin	450	9.77

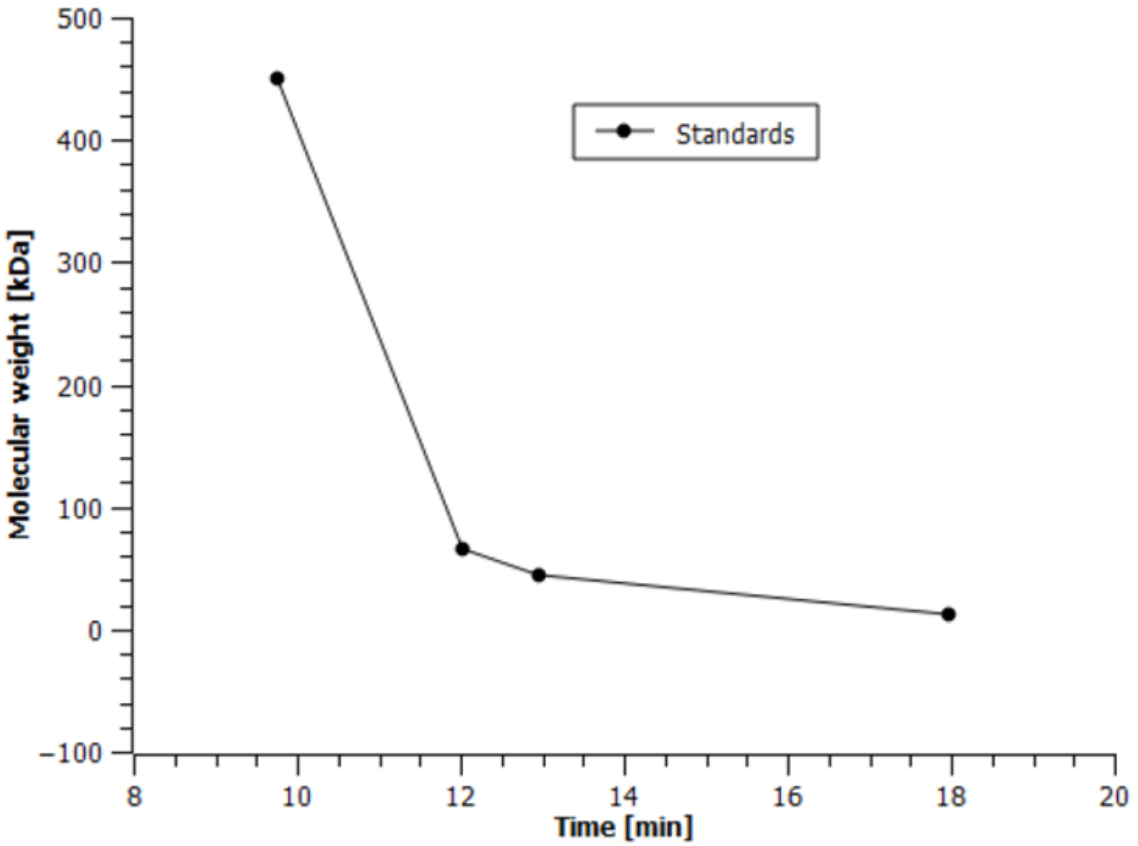

Figure 31: HPLC-SEC results for standards Ferritin 450 kDa, BSA 66 kDa, Ovalbumin 45 kDa, Cytochrome C 12 kDa with molecular weight on the y-axis.

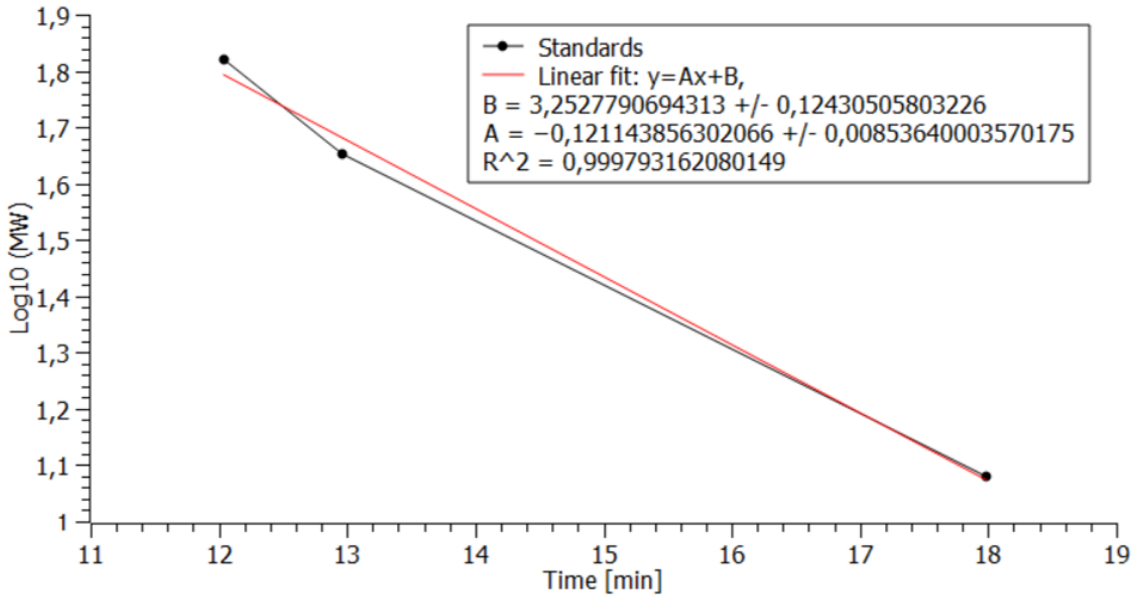

Figure 32: Standard curve for the HPLC-SEC using standards BSA 66 kDa, Ovalbumin 45 kDa, Cytochrome C 12 kDa.

Table 13: HPLC-SEC results on peak molecular weight of SPP and hydrolysates based on the linear standard curve in figure 32.

Sample	Time [min]	log(mw)	Molecular weight [kDa]
SPP	13.5	1.617337009	41.43210602
	16.57	1.245425371	17.59646259
	17.053	1.186912888	15.37846144
	19.023	0.948259491	8.876862462
Hydrolysate A	16.586	1.243487069	17.51810273
	17.048	1.187518607	15.39992505
	19.035	0.946805765	8.847198364
Hydrolysate B	16.581	1.244092788	17.54255263
	17.052	1.187034032	15.38275176
	19.048	0.945230895	8.815174111
Hydrolysate C	16.583	1.2438505	17.53276858
	17.052	1.187034032	15.38275176
	19.055	0.944382888	8.797978323
Hydrolysate D	16.586	1.243487069	17.51810273
	17.053	1.186912888	15.37846144
	19.049	0.945109751	8.812715514
Hydrolysate E	16.572	1.245183083	17.58664847
	17.044	1.188003183	15.41711751
	19.023	0.948259491	8.876862462
Hydrolysate F	16.58	1.244213932	17.5474467
	17.05	1.187276319	15.39133601
	19.047	0.945352038	8.817633394