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Characterisation of free and conjugated protease inhibitors from *Solanum tuberosum*

Kristoffer Lundmark

UPPSALA
UNIVERSITET

Teknisk- naturvetenskaplig fakultet
UTH-enheten

Besöksadress:
Ångströmlaboratoriet
Lägerhyddsvägen 1
Hus 4, Plan 0

Postadress:
Box 536
751 21 Uppsala

Telefon:
018 – 471 30 03

Telefax:
018 – 471 30 00

Hemsida:
http://www.teknat.uu.se/student

Abstract

Characterisation of free and conjugated protease inhibitors from *Solanum tuberosum*

Kristoffer Lundmark

The main purpose of the master thesis project is to investigate the influence of selected serine protease inhibitors (SPI) on the catalytic action of the serine proteases chymotrypsin and trypsin, in a conjugated and non-conjugated state. The inhibitors included for this study were extracted from *Solanum tuberosum*, i.e.common potato. The purification method included in this study consist of crude extraction by mixer, followed by a salt-out procedure with ammonium sulphate. Further purification steps were cation exchange chromatography and, finally, gel filtration to obtain SPI of high purity. The purified sample was then characterized by SDS-page and kinetic activity measurement of trypsin and chymotrypsin action on synthetic substrate derivate, N-Benzoyl-DL-arginine-4-nitroanilide hydrochloride (BAPA) and N-Succinyl-L-phenylalanine-p-nitroaniline (SFpNA) respectively. The characterization showed inhibitory inactivation of both pancreatic proteases. This would indicate successful extraction of SPI.

To investigate inhibitory action in a conjugated state, either enzyme or inhibitor was immobilized onto aluminium oxide membranes. Then two different experimental setups were tested, called experiment 1 and 2. In experiment 1, the inhibitor was immobilized and the interaction was monitored from a retention shift of enzyme flow-through compared to a blank column, using detection at 280 nm of the enzyme. In experiment 2 the enzyme was instead immobilized and a mixture of inhibitor and substrate was circulated with monitoring of the catalytic activity. The main goal was thus to measure the effects on the kinetics in the conjugated state compared to enzyme and inhibitor in the free state. The result from both experiment 1 and 2 did not yield consistent and reliable result so the discussed method should be regarded as preliminary results.

The study also includes investigation of inhibitor-enzyme interaction as revealed by molecular mass data to determine complex formation. This part was conducted with static light scattering analysis to determine the stoichiometry for the interaction between pancreas proteases and the inhibitor. Results from light scattering showed promising indication of many-to-one interaction between enzyme and inhibitor, which have been seen by previous studies. It should be considered a preliminary result as complex formation does not exclude aggregation of enzymes or inhibitor in the solution.

Handledare: Gunnar Johansson
Ämnesgranskare: Mikael Widersten
Examinator: Jan Andersson
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Karaktärisering av lösta och konjugerade serinproteasinhibitorer från *Solanum tuberosum*

Kristoffer Knut Johan Lundmark

Populärvetenskaplig sammanfattning

Proteaser är enzymer som bryter ner proteiner, nedbrytningen sker genom att enzymerna katalyserar klyvning av peptidbindningen i peptidkedjan via en hydrolys, d.v.s. en reaktion där vatten binder in där molekylen klyvs. Proteaser är mångfasetterade proteiner där olika grupper av enzymer har erhållit specificitet att bryta ner olika proteiner eller klyver proteiner med mer eller mindre hög specificitet för vissa aminosyror och är aktiva i olika miljöer. Mångfasetteteringen hos proteaserna har erhållits till största del som försvarsmekanism mot infektiösa arter och nedspjälkning av föda, dessa funktioner är livsviktiga för kroppen. Proteaser kan även ställa till med problem när de bryter ner fel sorts proteiner, nämligen kroppens egna proteiner, eller när infektiösa arter använder proteaser för att bryta ner kroppens barriärer och immunförsvar som skyddar mot angrepp. Därför har troligtvis alla arter utvecklat någon from av proteasinhibitor med egenskapen att stänga ner den katalytiska aktiviteten hos proteaser. Inhibitorerna används främst för att stänga ner katalytisk aktivitet mot angripande proteaser från infektiösa arter och förhindra att kroppens egna proteaser anfaller kroppen.

Proteasernas användning i naturen har väckt stort intresse inom forskningsvärlden och förståelsen över dess funktioner har stadigt börja framträda i och med forskningsframstegen. Med den ökade förståelsen har flera applikationer och användningsområden med proteasinhibitorer börjat framträda. Applikationer som att hindra den katalytiska aktiviteten när köttätande bakterier angriper, tillverka blöjor med inhibitorer som motverkar katalytisk aktivitet från tarmproteaser som kan irriterar huden. Det finns också applikationer inom akademin och industrin, där en kontrollerad katalytisk aktivitet kan användas i renings-/bevaringsprocedurer inom bioprocessing.

Examensrapporten vill därför undersöka effekten av utvalda serinproteasinhibitorer på tarmproteaserna trypsin och α-chymotrypsin. Serinproteasinhibitorn är extraherad från naturlig matpotatis. Det intressanta inslaget är inte bara att mäta den inhibitoriska aktiviteten från inhibitorerna fria i lösning, vilket har gjorts tidigare, utan även undersöka effekten av att binda inhibitorerna på aluminiumoxidmembran och undersöka hur det påverkar interaktionen. Det ska även undersökas om inhibitorerna interagerar bipolärt med enzymerna, det kommer att göras via ljusspridning vilket skulle stärka tidigare modeller som postulerar att två enzymmolekyler binder till en inhibitor. Rapporten kommer även att beskriva framreningen av inhibitorerna från potatis.

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Abbreviations

Aa – Amino acid

AlOx – Aluminium Oxide

BAPA – N-Benzoyl-DL-arginine-4-nitroanilide hydrochloride

CEC – cation exchange chromatography

LS – Light scattering

PSPI – potato serine protease inhibitor

RI – refractive index

SEC – size-exclusion chromatography

SFpNA – N-Succinyl-L-phenylalanine-*p*-nitroanilide

SPI – Serine protease inhibitor

Background

Enzymes

Enzymes are macromolecules which serve as biological catalysts. A catalyst increases the reaction rate for a chemical reaction by lowering the activation energy for the transition state between reactant and product, thereby promoting the reaction, see Figure 1. Most enzymes are protein based and many have bound metal ligands or other kinds of ligands bound to the protein surface to promote the catalytic properties (Nelson and Cox 2008). The structure of enzymes is often globular with an active pocket, which is a formation on the protein surface that has a specific geometry to fit the substrate in a way that promotes the reaction. Because the structure is an important factor in the functionality of enzymes they are often optimally active at a particular temperature and pH (Nelson and Cox 2008).

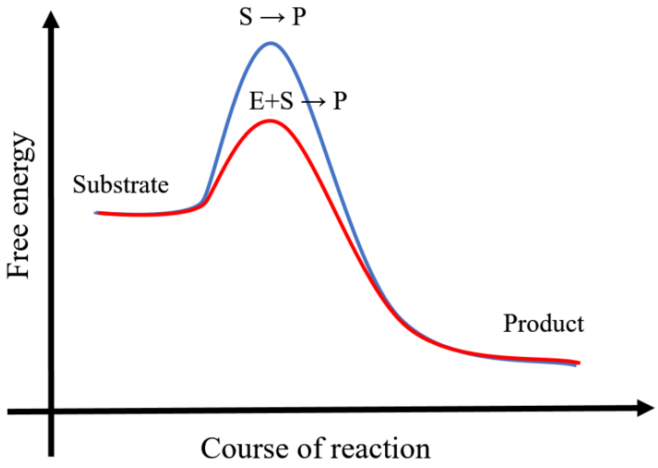

Figure 1. Conceptual graph of free energy during reaction with and without enzyme involved. E+S→P is the reaction complex with enzyme and S→P is the reaction complex without enzyme. The picture illustrates how the enzyme reduces the activation energy for the reaction, resulting in increased reaction rate.

Most enzymes increase the reaction rate by binding to the substrate, which was described earlier in Figure 1, thus the stipulation that reaction rate is dependent on substrate concentration. Therefore, a common mathematical model to describe enzymatic reaction is by Michaelis-mentens-kinetics which is described in Eq. 1. The Michaelis-mentens model can be applied directly to simple one step enzymatic reactions. Here, v_o is the reaction rate for the given reaction with fixed condition, V_{max} is the maximum reaction rate for the given enzyme concertation and other environmental conditions, [S] is substrate concentration and K_m is the substrate concentration at a reaction velocity of half V_{max}.

$$v_o = \frac{V_{max}[S]}{K_m + [S]} \quad (Eq. 1)$$

For more complicated enzyme cascades and reactions, where enzymatic reaction for instance have several steps or is effected by inhibition/allosteric regulation, other models are required to adjust for that kind of effect. For competitive inhibitors Eq. 2 can be applied to describe the enzymatic reaction, were the initial reaction rate is dependent on the concentration of both inhibitor and substrate. The kinetic model that is applied in this thesis project is for competitive inhibitor interaction Eq 2. Where v_o, V_{max}, [S] and K_m is the same as in Eq 1, [I] is the inhibitor concentration and K_i is the dissociation constant for inhibitor-enzyme complex.

$$v_o = \frac{V_{max}[S]}{K_m\left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (Eq. 2)$$

Proteases

Proteases or peptidases are a group of enzymes which start the catabolic process of cleaving proteins by cutting the peptide bond between the amino acids. This is done in a reaction called hydrolysis, which is a reaction where the chemical bond is broken and a water molecule is added instead of the previous bond, see Figure 2. Proteases have evolved multiple times and therefore use several kinds of mechanisms to hydrolyse proteins and can be found in all biological organisms and systems ranging from viruses to mammals (Oda 2011). To generalise, there are seven distinct protease groups, namely serine-, cysteine-, threonine-, aspartic-, asparagine-, glutamic- and metallo-proteases (Oda 2011). The name for each group comes from the functional residue that promotes a nucleophilic attack on the carboxyl carbon on the peptide bond. Figure 3 shows, as an example, a protease enzyme performing its catalytic action on a peptide bond.

Proteases play an important role in nature, the main usage of peptidases is to make nutrients accessible, defend against invasive species and attack proteins from invasive species. Humans use proteases to digest proteins and as key components in the immune system. Protein digestion takes place in the intestine and stomach. Meanwhile many proteases are used to digest foreign allergens and bacteria, while caspases for example plays an important role in apoptosis and uses cysteine amino acids to initiate its proteolytic activity and are targeting a four amino acid motif and cleaves after an aspartic acid (Thornberry and Lazebnik 1994).

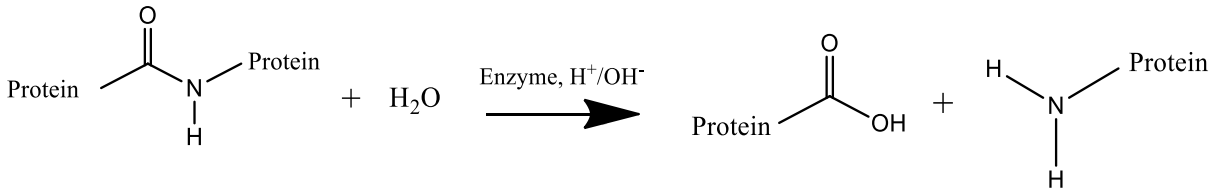

Figure 2. Reaction for hydrolysis, which peptidases use to cleave the peptide bond and add a water molecule between the bonds.

Proteases cause several problems when ending up in the wrong place, for instance it causes skin irritation/damage to epidermal tissue and are coupled to several diseases and infections. Example of disease is “acute pancreatitis” which is caused by pancreatic proteases. Due to obstruction the pancreas enzyme are activated prematurely and catalyses the degradation of pancreas tissue. This causes great pain and organ damage, which can be fatal in some cases (Nelson and Cox 2008). Other industrial problems can be shortening of food lifetime or lab cultivating where it hinders growth for bioprocessing.

Proteases also have a lot of application values, like food treatment and science reagents. Some applications can be regulating the fermentation process, tenderising meat and sample treatment, by destroying specific impurities or contaminates. Therefore, there is an increasing interest of understanding protease specificity and catalytic activity also including protease inhibitors to rigidly control the proteases reaction. Protease inhibitor can also be used in treating a various degree of diseases, and therefore could be used in diseases where own proteases attacks epidermal cells, or as an antiviral treatment.

Pancreatic serine proteases

Trypsin and chymotrypsin have an important role in the history of enzymology as trypsin was one of the first isolated enzymes by crystallization from urea by James Summer in 1926, he postulated from his experiment that all enzymes were proteins (Nelson and Cox 2008). This did however not gain acceptance until 1930 by John Northrop and Moses Kunitz, which also crystallized several enzymes including trypsin and pepsin and could also draw the conclusion that all enzymes were proteins (Nelson and Cox 2008). Therefore, these enzymes play an important role drawing conclusions one what an enzyme is.

The enzymatic activity from intestinal extract was first proposed in 1875 by author Heidenhain. The term "tryptic ferment" was some years later proposed by other authors (Vernon 1901). The concept of zymogens in the "tryptic ferment" was also postulated already in 1882 by Langley (Vernon 1901). Vernon in 1901 finally proved the idea of zymogen of the tryptic ferments, by extracting the pancreas gland and only observing ferment in gland extract exposed to active extract. Today this is a well-known process where active trypsin in the intestine digest parts on the zymogens secreted from the pancreas to activate the enzymes. Even during this period there was a postulation of more types of enzyme in the intestinal tract in addition to trypsin. This was not proven until 1934 when chymotrypsin was isolated by Kunitz and Northrop (Kunitz & Northrop 1934). Components that are well known today, namely Chymotrypsinogen, π-chymotrypsin and α-chymotrypsin were isolated by Kunitz in 1938 (Kunitz 1938). Chymotrypsin and trypsin structure and mechanisms have after this been extensively investigated. In 1940, catalytic action on a new substrate was in the forefront of research areas concerning these proteases. In the 1970-1980s the catalytic mechanism and amino acid sequencing was of the highest interest. During the 1990s investigation of inhibitors-enzyme interaction was carried out and crystal structures of bovine chymotrypsin was captured in 2 Å resolution (Frigerio *et al.* 1992).

What characterizes serine proteases is the utilisation of serine to promote catalytic degradation of peptide bonds, the serine proteases in this study are the pancreas serine proteases, trypsin and α-chymotrypsin. What characterizes serine proteases is the use of a "catalytic triad", a region consisting of three amino acids, namely aspartic acid, histidine and serine. The catalytic ability is promoted by a hydrogen bonding network described as a "charge relay system", where the electron density of the aspartate residue is partially transferred to the serine, thus making this residue more nucleophilic. This will promote serine to make a nucleophilic attack onto the carbonyl carbon at the peptide bond on the target protein. These proteases also include the distinct region which is called the "S1 domain", this region promotes peptide-target specificity by binding to a particular group of amino acids which exposes the peptide bond on the carboxylic side of that amino acid to the catalytic triad. The specific function of the S1 domain for trypsin and chymotrypsin is described below.

Pancreas serine proteases have evolved to be translated as a slightly longer inactive protein, the inactive proteins are called zymogens and represent a typical architecture for most pancreas proteases. The features of not being synthesized in the active form are essential for the enzyme in order not to digest the secretion tissue, therefore the activation is performed in the duodenum, initially by enteropeptidase which cleaves away two amino acids from the N-terminal of trypsin. When trypsin is active this starts a cascade event, where active trypsin activates more trypsin and also cleaves other zymogens to get active chymotrypsin and elastase.

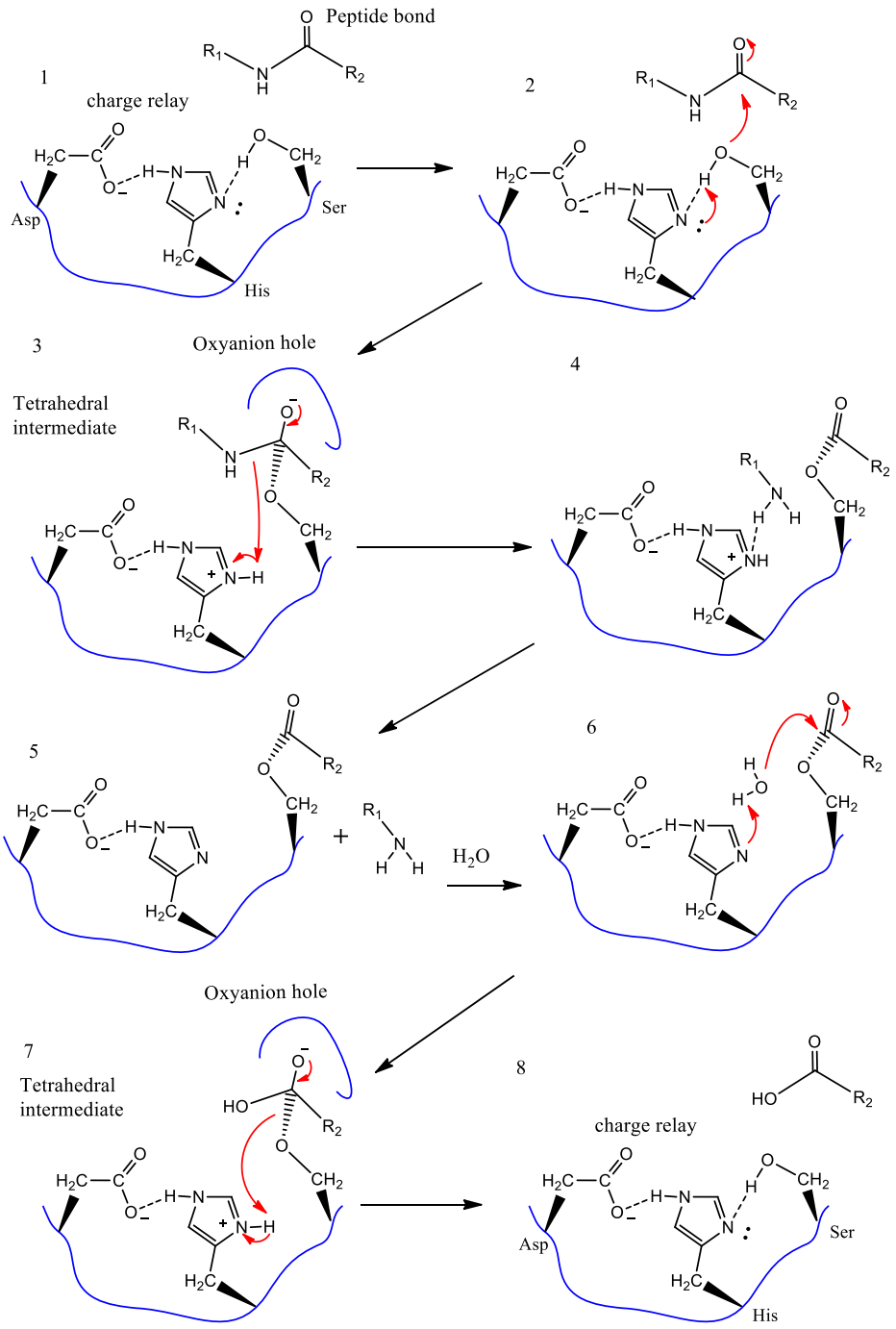

Figure 3. The reaction mechanism for the catalytic action on peptide substrate with a serine protease. (1) Shows the meeting between active catalytic triad and peptide bond. The alcohol group is very reactive due to weakening the hydrogen oxygen interaction with the Asp/His amino acid. (2) Oxygen in the serine alcohol group makes a nucleophilic attack onto the carboxylic side on the peptide bond, while hydrogen jumps to histidine group in the catalytic triad. (3) Tetrahedral intermediate is formed when oxyanion hole surrounds the oxygen in the carbonyl group in the peptide bond. This intermediate is formed to stabilize the negatively charge oxygen in the carbonyl, while hydrogen in the histidine from the catalytic triad will form an intermediate with the amide group on the peptide bond. (4) Shows the cleaved peptide bond where the amide side interacts with the histidine on the catalytic triad while the carboxylic carbon is bond to serine in the catalytic triad. (5) The N-terminal of the protein dissociate from the enzyme. (6) Water molecules are added when histidine from the catalytic triad and carboxyl group on the peptide interact with the hydrogens on the water molecule. (7) The tetrahedral intermediate is formed again, this will reset the enzyme by making the carboxyl group leave the enzyme, which makes the hydrogen on histidine to form a complex with the serine oxygen. (8) Enzyme can now catalyse another peptide bond as the active site is opened and hydrolysis completed.

Trypsin

Trypsin has a molecular weight of 23.3 kDa, 223 aa, and a pH optimum for catalytic activity at pH 7-9. Trypsin is one of the common pancreas serine proteases which cleaves on the carboxyl side of arginine and lysine residues which is promoted by the S1 domain, which is mostly hydrophobic except a negatively charged aspartate, which is characteristic for the S1 domain for trypsin. The negative charge aspartate will attract the positive end charge on arginine and lysine, also the long hydrophobic sides will promote fitting with the hydrophobic tail on arginine and lysine.

Trypsin has a high catalytic ability and self-digests itself in matter of hours if left in optimal conditions, therefore trypsin is secreted in early stages as trypsinogen, which is the zymogen of trypsin (Nelson and Cox 2008). Trypsinogen is 229 aa long and is about 25 kDa in molecular weight. There are two stages from zymogen to active trypsin is formed. First stage is to cleave the K6-I7 region in the trypsinogen with enterokinase, transforming the protein to the precursor β-trypsin. Next stage is to cleave the K131-S132 region by autolysis to end up with α-trypsin. The first stage takes place in the duodenum after hormone signalling food intake. The second stage is taking place in the intestinal tract and a similar process is used to activate other proteases.

Chymotrypsin

Chymotrypsinogen is the precursor to active chymotrypsin with 245 aa in initial length and after two precursor modification, where four aa are removed, namely aa 14-15 and aa 147-148, active chymotrypsin is produced. Common characteristic of chymotrypsin is the catalytic triad located at H57, D102 and S195, the oxyanion hole is located at G193 and S195 (Kraut 1977). This is one of the more complex and well understood proteolytic functions today.

Chymotrypsin has a molecular weight of 25 kDa and a pH optimum for catalytic activity at pH 7.8. Chymotrypsin is one of the common pancreas serine proteases which cleaves after all bulky hydrophobic side chains like phenylalanine, tyrosine, tryptophan and methionine. The specificity towards these amino acids is found in the S1 domain in α-chymotrypsin, which only is a deep hydrophobic pocket which ensures mostly large hydrophobic groups can fit.

Inhibitors

Dedicated protease inhibitors are often small peptides which interact with the enzyme to hinder the catalytic mechanism of the enzyme. Therefore, inhibitors are often optimally fitted to interact with the surface of a particular enzyme. The reason for an optimal fit is to prevent the inhibition of other enzymatic reactions in an organism so often inhibitors have very conserved regions which have evolved to bind to the particular enzyme with high specificity.

The inhibitor-enzyme interaction that is focused in this thesis project is action of inhibitors which bind directly onto the active pocket of the enzyme. This interaction will block the substrate to bind into the active pocket. Therefore, inhibitors with this behaviour are called “competitive inhibitors”, because they compete with the substrate to bind to the active pocket. Competitive inhibitors often have higher affinity to bind to the active pocket then the substrate, and thus outcompetes the substrate.

Kunitz-type serine protease inhibitors

In the "standard mechanism" with Kunitz-type inhibitors, the inhibitor binds directly onto the active pocket with an active structure often called "reactive loops" (Meulenbroek *et al.* 2012). The Kunitz-type SPI family inhibitory action is one of the most investigated mechanism for protease inhibition, as Kunitz-type protease inhibitors are a crucial defence mechanism to hinder the enzymatic activity for serine proteases (Ryan 1990) and has several key characteristics which are typical for the serine SPI family. The reactive loop, can either consist of one reactive (single headed) or two reactive loops (double-headed), which inhibits the enzyme by binding tightly to the active pocket with the reactive site.

The mechanism where protease inhibitors interact and inhibit is well understood. Protease inhibitors arose many times in evolution, which can be seen by the fact that 48 different families have to this day been discovered (Rawlings *et al.* 2004). But as many inhibitors evolved separately from each other there are many similarities between them, even when the amino acid sequence is very different (Otlewski *et al.* 2005). This thesis work worked with a non-covalent bound Kunitz-type inhibitor which interacts substrate-like with the enzyme. Interestingly, the inhibitor is not hydrolysed by the protease, which is the protease main purpose, to break down proteins such as the inhibitor. The serine protease inhibitors from potato interacts with the enzyme using the "standard mechanism" of inhibition. (Rawlings *et al.* 2004). The "standard mechanism" is often called the canonical mechanism, where the P_1-P_1' region located in the middle of the reactive loops, is hydrolysed by the enzyme, which creates a modified inhibitor. This modified inhibitor is prone to re-engage with the active pocket as the unmodified inhibitor (Laskowski and Qasim 2000). When the inhibitor interacts with enzyme again, the modified inhibitor will interact in a way that slows down the deacylation step, in the acyl-enzyme intermediate. This will favour the slow resynthesis of the peptide bond instead of hydrolysis mechanism, and the inhibitor is returned to its unmodified configuration (Laskowski and Qasim 2000). The name canonical comes from the fact that loops around P_1-P_1' region, will form a canonical structure of hydrogen bonds with the enzyme. This intermolecular structure stabilizes the enzyme-inhibitor interaction, which promotes the resynthetization. The canonical architecture is suggested to make the inhibitor rigid when confirming enzyme-inhibitor complex and displacing hydrolytic water molecules to interact with the amine on the inhibitor, and thus increasing the likelihood of resynthetization (Otlewski *et al.* 2005)(Krauchenco *et al.* 2003). The canonical mechanism is very common strategy for inhibitors, as 18 families of SPI have been discovered to this day, which also seems to have a common trait of inhibiting cysteine- and metalloproteases as well (Otlewski *et al.* 2005).

Potato tubers include a crucial defence mechanism against protein digestion by self, fungal and bacterial proteases (Valueva *et al.* 2000), where two homologs of serine protease inhibitors with PI of 5.2 and 6.3 and a molecular mass of 20 kDa were characterised. PSPI consist of two subunits, namely a 16.4 kDa and a 4.2 kDa subunit. The subunits are held together by two disulphide bonds. The configuration of the reactive loop for PSPI is not fully understood, but some authors suggests that it represents the two headed reactive group type (Meulenbroek *et al.* 2012).

Solanum tuberosum

Solanum tuberosum, commonly known as household potato, has been chosen as the source for SPI. This is based on that potato is a common food item which is inexpensive and contains high amounts of protease inhibitors. Actually, the majority of protein in potato tuber consist of protease inhibitors (Pouvreau *et al.* 2001) who found that 50% of total protein content in potato tubers were protease inhibitors. The most abundant is the Kunitz-type serine protease inhibitors, shorten to SPI in this report, which corresponds to about 20% of the protein in potato (Pouvreau *et al.* 2003).

Purification

Protein precipitation

Protein precipitation is based on changing the properties of the solvent to make the protein less soluble without changing its intrinsic properties, thus making it to aggregate from the solution. The method is essential as a simple starting step to purify and separate proteins from contaminates and also is a good method to preserve protein as they are protected from bacterial contamination and proteolysis (Scopes 1993). The most common method is salting out, which uses neutral salt as ammonium sulphate to react with polar areas on the protein, which exposes hydrophobic patches and thus make the protein to aggregate and separate from the solvent. This principle of salting out can be formulated by the Cohn equation, see Eq. 3. The Cohn equation where S is the solubility of the protein, B the ideal solubility, K is the salt specific constant and I is the ionic strength (Scopes 1993).

Cohn Equation:

$$log\ S = B - KI \qquad (Eq. 3)$$

Ionic strength can be calculated by, were c is ion concentration and z the ion charge:

$$I = \frac{1}{2} \sum_{i=1} c_i\, z_i^2 \ (Eq. 4)$$

Cation exchange chromatography

Ion exchange, shorten to IEX in this report, is based on separating particles based on the surface charge on the particle. Here, particles with opposite charge to the resin will bind to the stationary phase, until the particle is being eluted by changing conditions, commonly a change in salt concentration or pH (Scopes 1993).

Cation exchange chromatography (CEC) absorb or rather interact with positive charged molecules. The method is based on negative charged resin, which uses intermolecular forces to attract the positively charged molecules to adsorb to the surface of the column. After the molecules have adsorbed to the surface the molecules can be released by high salt concentration, which out competes the molecules, or high pH which will change charge on amphoteric ionic molecules, like proteins. Protein net charge is dependent on pH, where the isoelectric point (pI) will give neutral net charge; below pI the proteins have positive net charge and above pI the protein has negative net charge.

Size-exclusion chromatography

Size-exclusion chromatography (SEC in this report) is based on highly porous beads. There is a variety of matrices like agarose, polyacrylamide or dextran, just to name a few. The different types of matrices create different pore sizes. The pore in the beads will separate particles in the mobile phase based on the size of the particles which in most cases will result in a separation also based on molecular mass. But in principle large particles will elute first and smaller molecules later, due to time delay caused by that small molecules enters the pores more frequently than larger molecules. This is because of the pores in the beads will reversibly accommodate particles which can fit into the pores on the surface, thus slowing the particles movement through the column. The mobile phase is basically any buffer that suits downstream processing and protein stability; the only criteria for the mobile phase is not using to extreme acid or basic environment.

In the parallel purification strategy utilised for this project, this is the later step which will separate proteins after molecular weight, or more correctly size. In this project two different columns were utilised ranging from 450 ml (BV) Bio-Gel P-100 from Bio-Rad laboratories and 30 ml BV 200 Superdex from GE healthcare, which is a polyacrylamide and agarose-dextran matrix respectively.

Concentration of protein samples

For analytic proposes it can often be required to concentrate the protein solution. This is done by removing buffer while keeping protein in the solution, until desired concentration is achieved. The most common method is either to use dialysis tubes or ultrafiltration membranes. Both methods utilise a cellulose membrane with a cut-off value around 1 kDa, meaning that molecules below 1 kDa in size will travel through the membrane, while bigger molecules, like proteins, will not go through the cellulose membrane. In this project both dialysis tubes and ultrafiltration were used. Main difference is that dialysis tubing procedure employs dry Sephadex beads as an "outside" water absorbent, forcing diffusion of buffer through the tube while the membrane uses pressure to force buffer through the filter. Therefore, the ultrafiltration method is often faster and easier to use.

Aluminium oxide membrane

Immobilization of SP and SPI was performed onto aluminium oxide membrane. Aluminium oxide, in this report shorten to AlOx, are commonly used as an inorganic micro-filter, which has been treated in a sensitive way in acid to create small pores about 0.02-0.2 µm in diameter and are chemically stable between pH 4.5 to 9.5. Aluminium oxide membrane is very porous and brittle, which makes it difficult to produce and work with as they are very fragile.

Immobilization of protein ligands onto aluminium oxide

In the project the AlOx membrane used was Anodisc™ from Whatman™, which is an industrially produced membrane with chosen pore diameter of 0.2 µm. Immobilization is based on an initial deprotonation of the aluminium oxide with Triethylamine, see figure 4.

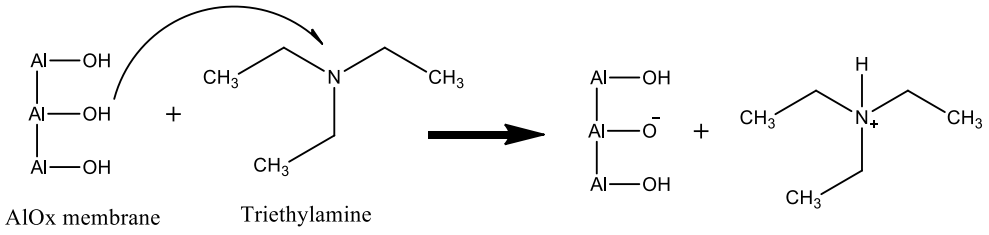

Figure 4. Reaction mechanism were Triethylamine deprotonates the AlOx surface.

After the deprotonation the AlOx surface (3-aminopropyl)triethoxysilane is used to bind to the surface as a spacer, (3-aminopropyl) triethoxysilane is shorten to APTES in this report. APTES reacts with the more reactive oxygen on the surface by undergoing a nucleophilic substitution, where the tail is replaced with the aluminium oxide and also forming ethanol as a leaving group, see figure 5.

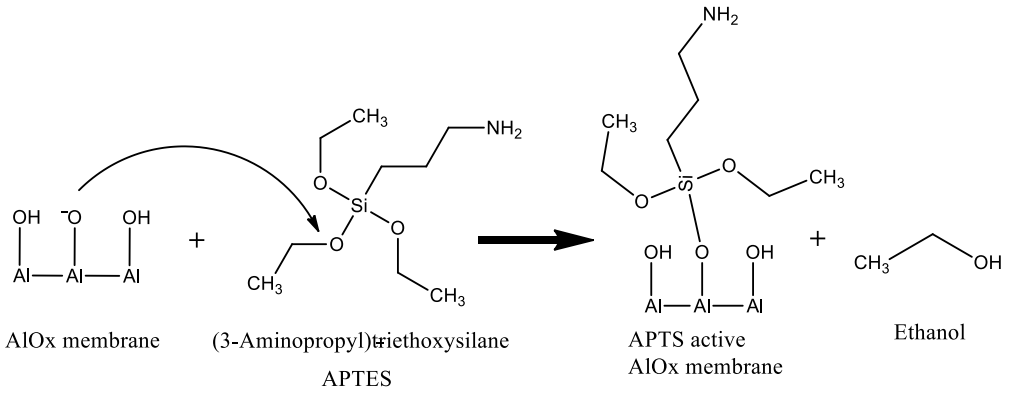

Figure 5. Reaction mechanism when the APTES spacer reacts with the deprotonated AlOx membrane by a nucleophilic substitution and a formation of ethanol.

To make the spacer reactive towards amino groups, that will enable proteins to bind to the spacer on the AlOx membrane, we employed CDI activation. CDI or 1,1-Carbonyldiimidazole will react with the terminal amine on the APTES spacer, where one imidazole molecule will leave the CDI molecule and the rest of the molecule will bind to the spacer. The newly modified spacer will be able to react with amino groups, that exist on for instance proteins. When an amino group reacts with the CDI active spacer the amine will substitute the remaining imidazole group with a protein ligand, which is done in a basic environment to promote a reaction, see figure 6.

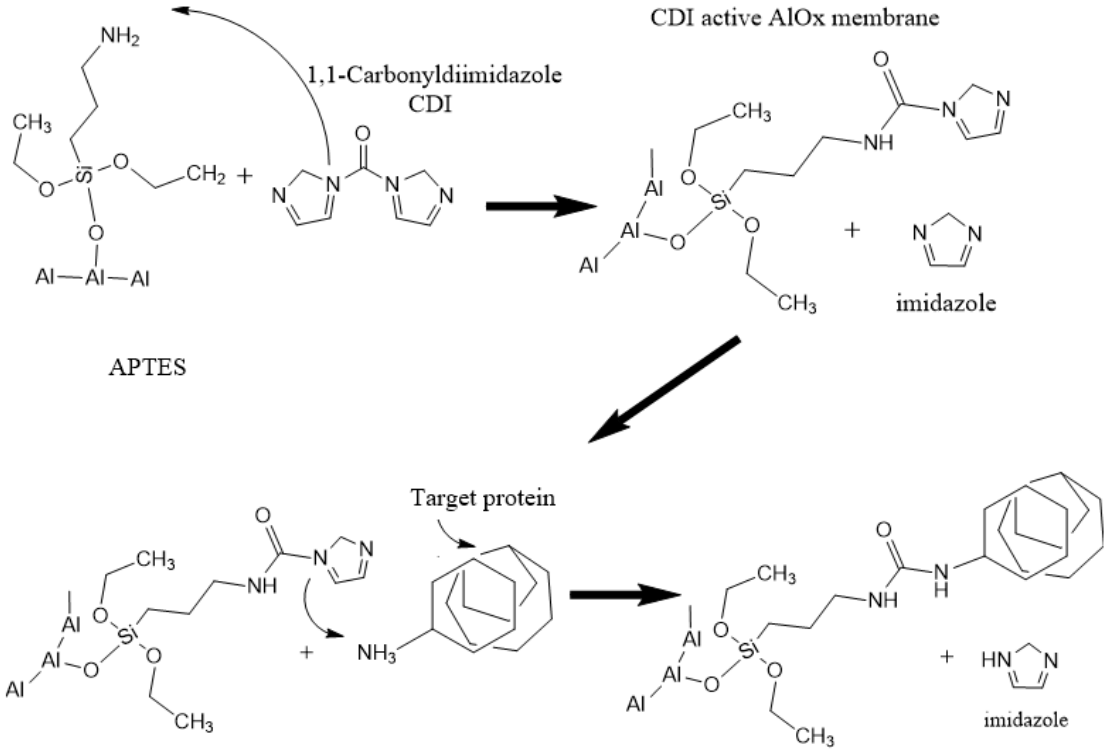

Figure 6. Reaction which CDI activates the spacer on the AlOx membrane and later promotes ligand binding by the same reaction as for the CDI with spacer reaction.

Characterization and catalytic measurement

Quantity determination

There are several methods available to determine concentration of proteins with varying degree of accuracy. This report will mainly be using Beer's law to calculate concentration from spectrophotometer measurement of absorbance at 280 nm, see Eq. 5. This method is in general a good enough method in most cases, but it is not the most accurate method and requires pure sample to get good estimation of the concentration. When measuring, it is important that absorbance is in the linear region around 0.04 - 1 absorbance units, (the upper limit may be higher depending on the instrument used) for accurate measurement. When using Beers law to calculate concertation an extinction coefficient is required, which has been derived theoretically or experimentally and describes how much a particular molecule absorbs at a particular wavelength. What actually controls the absorbance at 280 nm is the presence of aromatic rings in a molecule, so for proteins the primary reason for light absorption at 280 nm are the amino acids tryptophan, tyrosine and, to a lesser extent cysteine disulphide bonds and phenylalanine. In this project the SPI protein has a theoretically derived extinction coefficient from ProtParam, while the rest is experimentally derived, see table 1, for extinction coefficients used in this report.

Beer's law

$$A = \varepsilon\, l\, c \quad (Eq.5)$$

Calculation to convert the extinction coefficient from molar to weight.

$$\varepsilon_{percent} \cdot M_w = \varepsilon_{molar} \cdot 10 \; (Eq. 6)$$

Calculation for concentration with extinction coefficient in percent with 1 cm path length

$$c = \frac{A}{\varepsilon_{percent}} \cdot 10 \left[\frac{mg}{ml}\right] \; (Eq. 7)$$

Table 1. Extinction coefficients and wavelength chosen to calculate concentration during this project.

Protein/chemicals	Wavelength [nm]	Extinction coefficient molar [$M^{-1}cm^{-1}$]	Extinction coefficient percent [$1\ g/100\ ml\ cm^{-1}$]
Trypsin	280	33677	14.2
Chymotrypsin	280	51000	20.4
Elastase	280	52318	20.2
PSPI-P1	280	27430	11.4
PSPI-P2	280	27180	13.6
Nitroaniline	410	8800	-

SDS-page

SDS-page is a commonly used electrophoresis method where the proteins are completely denatured by the detergent SDS (sodium dodecyl sulphate) before electrophoresis to determine size of proteins. The denaturation and binding of DS-micelles will result in a uniform shape and charge density for all proteins. As a consequence, the separation in a suitable gel will depend chiefly of peptide chain length if any disulphides also are cleaved. This will provide an estimate of the chain length, and thus the molecular weight if a standard reference sample is included. The SDS-page experiment will be performed in the Phast-system.

Kinetic measurement

The most common way to measure activity is by detecting product formation continuously over time, I.e. real time assay. The most common method of measuring activity for serine protease trypsin and chymotrypsin is by using synthetic amino acid derivatives, which release a chromogenic substance if hydrolysed. The chromogenic substance colouring the solution with a specific colour, which can be used to quantitative measure the reaction rate. P-nitroaniline is a such chromogenic substance, which forms a yellow solution and has an extinction coefficient of 8800 M^{-1} cm^{-1} at 410 nm. In this project two well defined synthetic amino acid derivatives are used to assess the catalytic action of trypsin and chymotrypsin, namely N-Benzoyl-DL-arginine-4-nitroanilide hydrochloride and N-Succinyl-L-phenylalanine-*p*-nitroanilide, shortened to BAPA and SFpNA, respectively, in this report. Both substrates are chromogenic, forming p-nitroaniline molecules as a result of the hydrolysis, see figure 7 and figure 8.

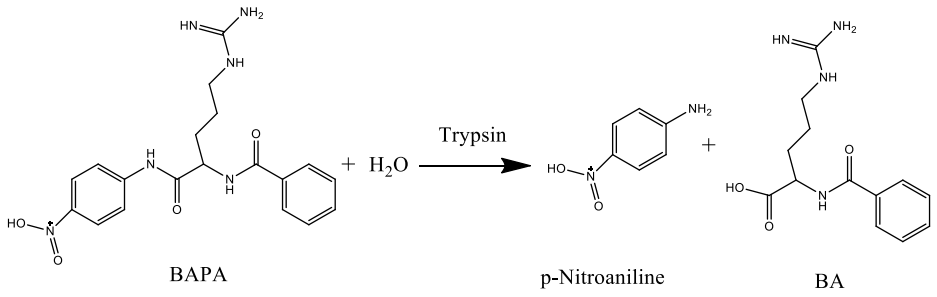

Figure 7. Catalytic action on chromogenic substrate BAPA with trypsin, to form p-nitroaniline, which can be detected by absorbance at 410 nm.

The product p-nitroaniline which is formed when substrate BAPA and SFpNA is hydrolysed can be detected from the absorbance at 410 nm as mentioned before. Therefore, it is common to add substrate and enzyme in a quartz cuvette and detect the increasing absorbance at 410 nm over time.

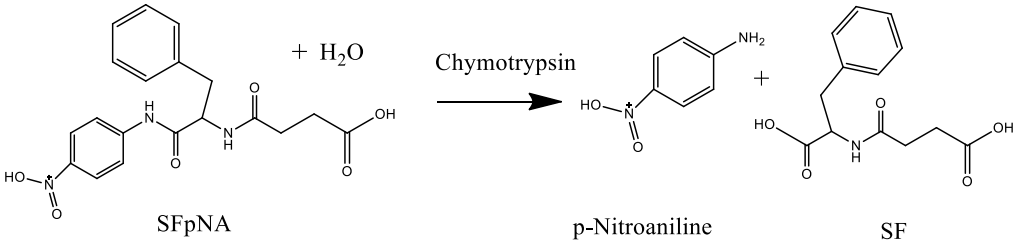

Figure 8. Catalytic action on the chromogenic substrate SFpNA with chymotrypsin to form p-nitroaniline, which can be detected by absorbance at 410 nm.

Light scattering

Light scattering (LS) is the principle of irradiating a sample with light at a particular wavelength and detecting the scattering from the medium. By normalizing the medium the scattering from irregular particulars in the medium can be detected, and by the change in intensity we can transform the response and derive the size and mass of the particle from the LS response. By now combining the LS response with refractive index detection, the concentration can be cancelled out by the formula described in the following, see Eq. 8, 9 and 10. for a schematic figure of light scattering and formula showing the relationship between light scattering, refractive index and molar weight.

Following approximation are made to calculate the molecular weight from measuring the light scattering as described above, followed by the change in refractive index caused by the irregular particles, see Eq. 8 and 9.

$$LS \sim c \cdot M_w \cdot \left(\frac{dn}{dc}\right)^2 \quad (Eq. 8)$$

From Eq. 8 one can see that light scattering (LS) is dependent on the concentration *c*, the molar weight M_w and change in refractive index (*dn/dc)* squared.

$$RI \sim c \cdot \frac{dn}{dc} \quad (Eq. 9)$$

From Eq. 9 one can see that refractive index (RI) is dependent on the concentration *c*, and change in refractive index (*dn/dc).*

$$\frac{LS}{RI} \sim M_w \cdot \frac{dn}{dc} \quad (Eq. 10)$$

When combining Eq. 8 and Eq. 9, to produce a quote between LS/RI, one can see that it is dependent on the molar weight and change in refractive index, which is stated by Eq. 10.

Methods

Purification of serine protease inhibitors

Experimental work focusing on the interaction of serine protease inhibitor with enzyme first required that the inhibitors were purified from common potato tubers. To purify the two homologs of serine protease inhibitors SPI-1 and SPI-2 the previously developed purification method (Eriksson 2016) was used with some modification for upscaling reasons.

Crude extraction of protein from *Solanum tuberosum*

Extraction of protein from *S. tuberosum* was started by homogenizing 1 kg *S. tuberosum*. Homogenization was performed in a kitchen mixer with potato tubers and 100 ml milliQ water until components were fully homogenized. The homogenate was then in cloth filtered under pressure until no more fluid could be extracted. The fluid was then collected and 0.2 % (w/w) of ascorbic acid was added (0.75 g) to prevent oxidation of the crude protein mixture. The sample was kept overnight in cold room in a 40 % saturated ammonium sulphate solution to prevent degradation of protein, therefore 91 g of ammonium sulphate was added to the crude protein mixture. The next day the mixture hade separated into two phases; a bottom precipitated layer and a top suspension layer. The top layer was stored, as a matter of safety, and the bottom layer (precipitate) was collected and resuspended in 200 ml 40 mM sodium acetate buffer, pH 4.6.

To separate protein components from cell and tissue components from the crude protein mixture, the mixture was centrifuged for 60 min at 5000 rcf (relative centrifugal forces) at 4 ^{o}C. After centrifugation, suspension was collected and heat shocked for 15 min in 56 ^{o}C. Heat chocking was used to terminate unwanted enzymatic activity, by denature heat sensitive enzymes. Thereafter, the sample was centrifuged 30 min at 5000 rcf at 4 ^{o}C and the supernatant was collected after centrifugation.

The last crude purification step included repeated precipitation of the protein sample by making another saturation to 40% with ammonium sulphate. The saturation was performed by adding 53.42 g of ammonium sulphate. 40% saturated protein sample was then centrifuged for 30 min and 5000 rcf. For later processing the precipitate was suspended in 25 ml of 40 mM sodium acetate with pH 4.6 and then filtered with 0.5 µm Millipore cellulose membranes.

Desalting protein extract with 40 % saturated solution of ammonium sulphate

To remove a high remaining concentration of ammonium sulphate from the redissolved protein sample a 500 ml bed volume of Sephadex G-25 column was used to perform buffer exchange to 40 mM sodium acetate buffer with pH 4.6. The column was equilibrated with 40 mM sodium acetate buffer with pH 4.6, which was also used to redissolved the protein sample. 20 ml of redissolved protein sample was injected into the column with a flow rate of 5.6 cm/h (8.37 ml/min). The first 125 ml flow through from the column was collected in one fraction, after that the eluate was collected in 8 ml fractions until 220 ml flow through was collected.

Cation exchange chromatography

First protein separation step is to separate protein with CEC. As mentioned earlier the two homologues of SPI-1,2 have a small difference in pI and thus also in net charge, and will therefore be expected to separate in this step. The CEC column was a 30 ml bed volume of CM-Sepharose FF. The column was equilibrated with 5 CV of 40 mM sodium acetate buffer with pH 4.6. The flow rate was then adjusted to 10.12 cm/h (0.53 ml/min). 22 ml desalted *S. tuberosum* protein sample was then injected onto the CEC column followed by1 CV of 40 mM sodium acetate buffer with pH 4.6 to elute nonbound components. Then the inlet was placed in a gradient mixer which created a salt gradient over an interval of 800 ml, the gradient was a 0-0.5 M NaCl in 40 mM sodium acetate buffer with pH 4.6. Next step is to “strip” the column with a 1 M NaCl in a 40 mM sodium acetate with pH 4.6 and finally introducing a 20 % ethanol solution for storage. 8 ml fractions were collected from the flow through

starting from sample injection to the "stripping" phase; in total 119 fractions were collected. Fraction absorbance was measured in a 96-well plate reader at 280 nm, see CEC result page 17.

Size-exclusion chromatography

To further purify each protein based on size a SEC column of 450 ml bed volume packed with Bio-gel P100 was used. The SEC column was first equilibrated with 100 mM phosphate buffer, pH 7.3. Fractions from the peaks in the CEC purification step were pooled and checked for inhibitory affect with trypsin using BAPA as substrate and monitoring at 410 nm. Peaks were then pooled and concentrated by ultrafiltration with a cut-off of 1 kDa and was concentrated 1.35x. 6 ml concentrated sample was injected onto the SEC column and 8 ml fractions were collected in a total of 672 ml eluate, giving 85 fractions, see result at page 18. All fractions were analysed for absorbance at 280 nm and observed peaks at that wavelength were further analysed with SDS-page. Pooled fractions containing possible SPI protein were then concentrated using dialysis tubes. SEC purification was done for both peaks that were eluted from the CEC step.

Immobilization of pancreatic proteases and SPI onto aluminium oxide membrane

To investigate the kinetic differences between enzyme-inhibitor interaction from a conjugated state and non-conjugated state, both the possible inhibitors from the *S. tuberosum* and trypsin and chymotrypsin were immobilized onto aluminium oxide membranes (AlOx). The AlOx membranes were first rinsed in 5x milliQ water, then 10 ml acetonitrile with 100 µl triethylamine and 250 µl APTS was added to the membranes and incubated for 1 h. Thereafter, the membranes were rinsed six times with ethanol and one time with acetone. The membranes were then baked in an oven for 10 min at 90 ^{o}C to dry away the acetone. The spacer amino groups were then activated with CDI (1-1-carbonyldiimidazole) by mixing 100 mg CDI and 100 µl triethylamine in 5 ml dry acetonitrile and finally adding the mixture onto the AlOx membrane to incubate the membrane for 1 h. The membranes were later rinsed three times with ethanol, four times with milliQ water and one time with acetone followed by drying for 10 min in the oven at 90 ^{o}C. For each immobilization onto membranes a total of 10 nmol of enzyme or inhibitor per membrane was used. Each enzyme was immobilized on 10 activated membranes whereas 5 membranes were used for each inhibitor sample.

Light scattering to investigate interaction between SPI acting on pancreatic proteases

Light scattering was used to investigate interaction with trypsin, α-chymotrypsin and elastase together with PSPI-P1 with a 40 mM TRIS buffer with pH 8.3. This experiment was done several times with different ratios but with a total volume of 1 ml, see table 2.

Table 2. Setup for experiment with trypsin or α-chymotrypsin as enzyme with PSPI-P1 as inhibitor, the experiment is setup with different concentration and ratios between enzyme and inhibitor which is described by the table.

Experiment	inhibitor concentration [µM]	enzyme concentration [µM]	Ratio enzyme: inhibitor
1	2	2	1:1
2	2	0.04	1:50

Kinetic measurements of catalytic activity of pancreatic proteases effected by SPI in non-conjugated state

The inhibition experiment was carried out in a 700 µl quartz cuvette with absorbance detection at 410 nm for 240 and 300 seconds for chymotrypsin and trypsin, respectively. The catalytic activity was measured by product formation, where catalytic action of the respective enzyme yielded detectable p-nitroaniline. Enzyme concentration was 2 µM for chymotrypsin and trypsin respectively. The following substrate concentration was used for respective enzyme experiment: 50 µM, 100 µM, 300 µM and 500 µM of BAPA and SFpNA. All concentrations of substrate were also tested with different PSPI-P1

concentrations, concentration used for each substrate concentration series was 0.1 µM, 0.2 µM, 0.5 µM and 1.2 µM with PSPI-P1.

Experiment 1 setup to determine catalytic activity for conjugated pancreatic proteases

To measure interaction between enzyme and immobilized SPI, an experimental setup was evaluated. This is due to the difficulty measuring the enzymatic activity with inhibitor directly in the AlOx membrane. The method is based on allowing free enzyme solution of trypsin and chymotrypsin with different concentrations move through the column at a flow rate of 100 µl/min. The column was holding five AlOx membranes with immobilized PSPI-P1, 400 µl fractions were collected from the column flow through. Between the column and the fraction collector was a spectrometer flow cell, which was measuring in real time at 280 nm absorbance during each experiment. For the experiment also several reference proteins were used, namely serum albumin, ribonuclease and myoglobin. Additionally, comparison experiments with blank column with five membranes of non-derivatized and APTS-CDI activated but Tris-capped AlOx membrane were carried out, for schematics see figure 9.

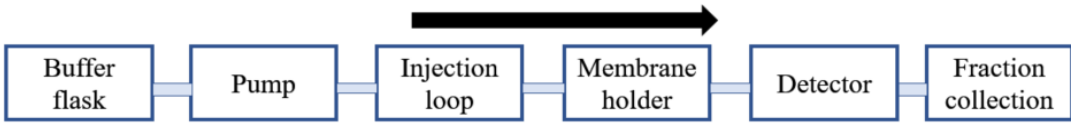

Figure 9. Schematic picture of the experimental setup for determine interaction between trypsin and α-chymotrypsin when passing bioreactor with conjugated serine protease inhibitor on aluminium membrane. Where the buffer is successively added by a HPLC pump while the sample is injected through a 200 µl injection-loop passing through 5 AlOx membranes in the membrane holder. The flow through from the membrane holder is then monitored at 280 nm adsorption using a detector, then collecting in 1 ml fractions.

Experiment 2 setup to determine catalytic activity for free proteolytic enzymes effected by conjugated inhibitors

An experimental method was validated. The method is based on allowing different concentrations 2 mM, 0.5 mM and 0.25 mM of free PSPI-P1 together with either 1 mM, 0.5 mM or 0.2 mM of BAPA substrate to circulate in a bioreactor holding 10 AlOx membranes with immobilized trypsin and measure the absorption at 410 nm continuously in a flow cell, see figure 10. For each experiment 200 µl sample was added and allowed to circulate in the system for 40 min while simultaneously gathering absorbance data in the flow cell at 410 nm. The flow rate of the system was set to 210 µl/min while the system volume is 2.4 ml.

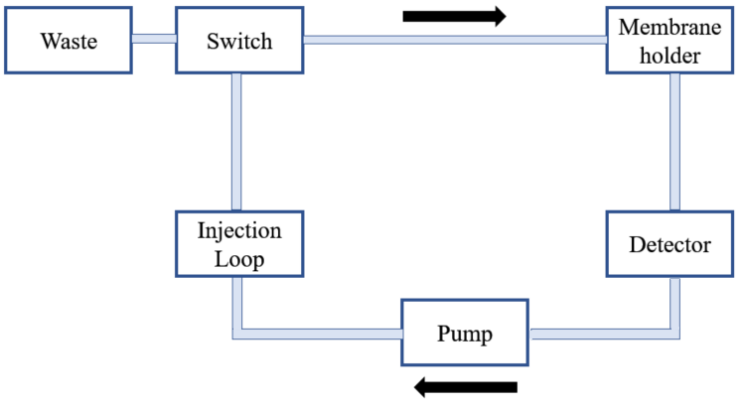

Figure 10. Experimental setup for measuring catalytic activity for trypsin when a solution of substrate and inhibitor is circulated through the bioreactor with trypsin immobilized on AlOx membrane. The injection employed a loading loop with a valve to inject sample into the closed system, the loop had a volume of 200 µl. The waste valve is used to replace system with new buffer after each experiment by directing the output to a waste beaker and input to the buffer flask. The membrane holder contains 10 AlOx membrane while the detector recorded the absorption at 410 nm for 2400 sec. The system has a total volume of 2.4 ml.

Result

Purification of serine protease inhibitor

After the initial extraction and precipitation, the extract was desalted and total protein content was measured to be 950 mg. Protein content was calculated by measuring absorbance at 280 nm and approximating 1 abs = 1 mg/ml, then multiplying the estimated concentration of 39.6 mg/ml with the volume of 24 ml.

Cation exchange chromatography

The first purification step was CEC, the crude extract proteins were separated based on charge properties by CEC, the corresponding chromatogram can be seen in figure 11. The chromatogram shows three peaks in the elution interval where the SPI target is expected to elute. Therefore, fractions from the observed peaks were pooled and concentrated, it should be noted that CEC purification was performed two times, in the first experiment the fractions were measured in 96-well plates at 280 nm while in the second experiment the fractions were measured manually in a 0.7 ml cuvette at 280 nm. From the first experiment the resolution from the 96-well plate measurement had excessive noise and peak two and three were merged together, therefore during the characterisation the report will only talk about peak 1 and peak 2 from the CEC step, and also the possible inhibitor will also only be called PSPI-P1 and PSPI-P2, were P1 and P2 comes from the CEC peak they were collected from, see figure 11. After pooling fractions containing the peaks the concentration was calculated, see table 3.

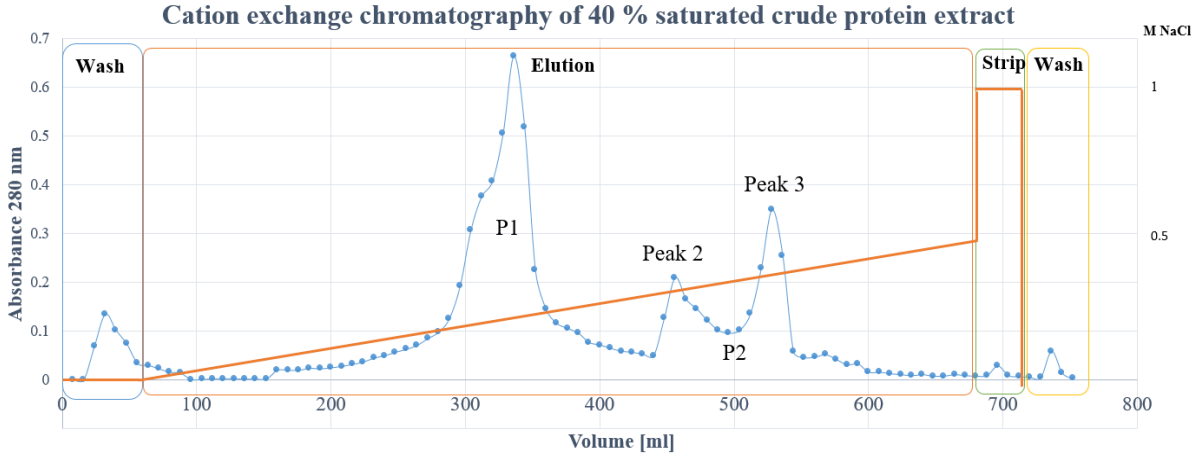

Figure 11. Chromatogram from measuring absorbance at 280 nm, measuring in 0.7 ml quartz cuvette of all the CEC fractions, which is shown with the blue line. Colour boxes describe different steps in the separation protocol where blue and yellow is the wash schedule, green is the final strip phase and elution phase is represented in the orange box. The orange line and secondary y-axis shows the change in NaCl concentration. P1, peak 2 and peak 3 is the peaks represented in the graph. It should be noted during collection peak 2 and peak 3 was pooled and the sample of peak 1 and peak 2 will be called P2, as shown in the graph.

After pooling of selected fractions, the pooled samples needed to be analysed for inhibitory activity against serine proteases and for protein components. Therefore 1 ml sample from both CEC-P1 and CEC-P2 was analysed separately with LS. The LS setup first separates protein by isocratic separation with a Sepharose 12 column. The resulting LS-data can be seen in figure 12 (right side). Collected fractions from the flow through of the LS-equipment was analysed by SDS-page, which was processed by silver stain due to low protein concentration, see figure 12 (left side). The result in the SDS-page for CEC-P1 shows four with protein bands corresponding to 50 kDa, 30 kDa, 25 kDa and 17.5 kDa. The CEC-P2 result shows bands at 66 kDa, 18 kDa and 22 kDa. It should be noted that the bands between 30 and 20 kDa can be the same protein for the CEC-P1 and CEC-P2.

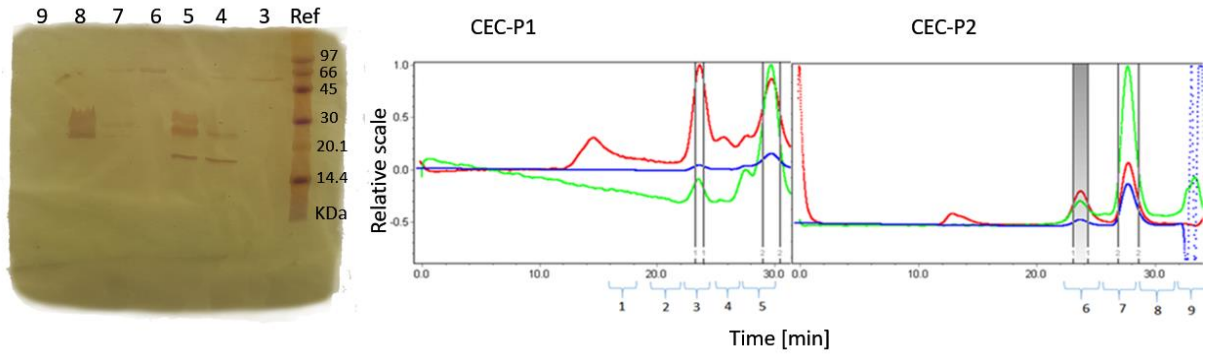

Figure 12. Left picture shows the silver stained polyacrylamide gel from the SDS-page. SDS-page containing CEC-P1 and CEC-P2 pooled sample that has been run through the LS setup. Fractions collected can be shown in the LS chromatogram, which is represented by the numbers under the x-axis, right picture. Chromatogram shows that absorbance at 280 nm as a green line, refractive index as a blue line and a red line represents the light scattering. The chromatogram is plotted with time in the x-axis and a relative scaling of all recorded parameters on the y-axis.

Table 3. Molecular weights from peaks from LS-data, the calculated molecular weight were derived from LS data and the calculation was performed in ASTRA software.

Peaks from CEC Chromatogram	Peaks in the LS	Mw [kDa]
CEC-P1	Peak 1	71.95 kDa
	Peak 2	17.15 kDa
CEC-P2	Peak 1	72.08 kDa
	Peak 2	15.69 kDa

Besides running CEC peaks through LS, the CEC peaks were tested for inactivation of catalytic activity for trypsin digesting BAPA. Experiment shows that both CEC-P1 and CEC-P2 peaks inactivated catalytic activity when 100 µl of either peak was used.

Size-exclusion chromatography

The second purification step was based on separation of proteins by size, this was done by the SEC method. Figure 13 shows the chromatogram from the first SEC, were absorbance was measured in a 96-well plate at 280 nm.

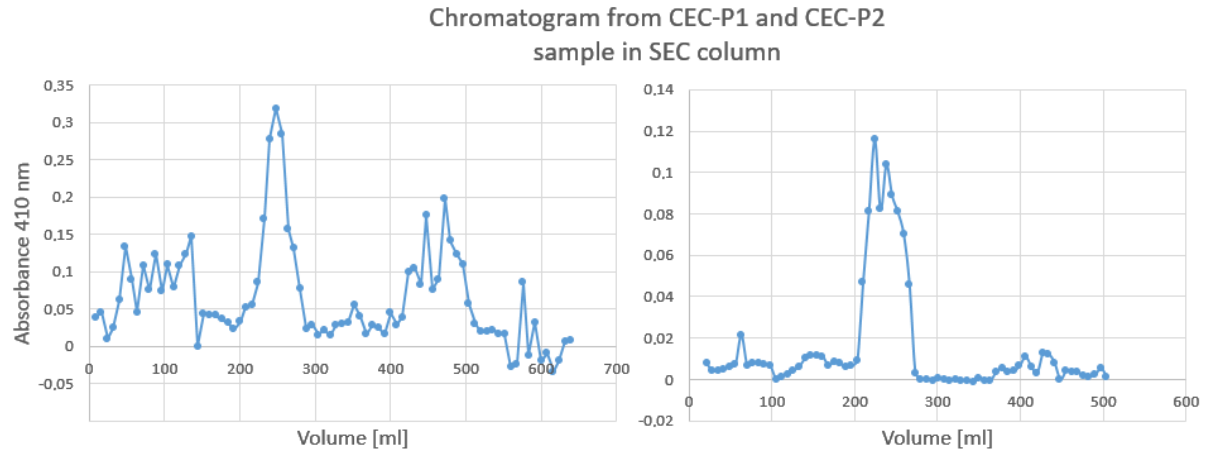

Figure 13. Isocratic SEC separation of protein from CEC-P1 (left picture) and CEC-P2 (right picture). Fraction from the SEC step have been collected in 8 ml fractions and measured absorbance at 280 nm.

The second SEC experiment was performed with simultaneous LS measurement for CEC-P1 fraction, note that no other of the three peaks was done with LS. The LS shows two charateristic peaks at 280 nm with corresponding scattering and refractive index peaks, these peaks were evaluated by the ASTRA 5.3.4 software.

After the contration step the purificated PSPI-P1 was tested for inhibitory action on trypsin, the result can be seen in figure 14, where the initial rate (the slope) clearly decreases with increasing inhbitor concentration. The final volume and absorbance measurement of the pools can be seen in table 4. After absorbance measurement, the concentration of both peaks from the SEC purificaiton was 24.9 µM for CEC-P1-SEC-P1 and 11.4 µM for CEC-P2-SEC-P1.

Table 4. Absorbance and volume collected from SEC chromatography fraction.

Peaks from SEC Chromatogram	Volume [ml]	Absorbance at 280 nm
CEC-P1-SEC-P1	8	0.678
CEC-P2-SEC-P1	3	0.313

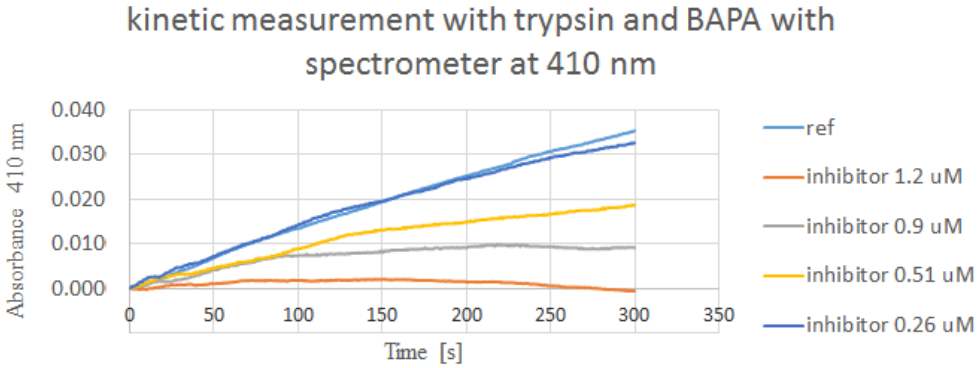

Figure 14. Kinetic measurement with BAPA and trypsin with various concentration of SPI. Which is plotted with absorbance at 410 nm vs time in sec.

For the second time the SEC experiment was carried out, another setup was tried where LS-machine was utilised together with a Superdex column for size separation, see figure 15. The data shows two clear peaks, named SEC2F1 and SEC2F2 in this report.

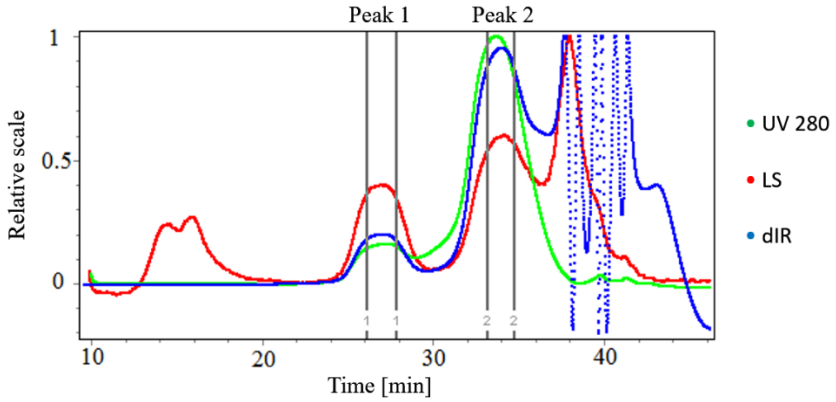

Figure 15. LS recording from isocratic SEC purification from CEC-P1 purified potato protein extract. The recording contains data about UV 280 nm (UV280, green line), light scattering (LS, red line) and refractive index (dIR, blue line), where data from all the parameters have been plotted onto a synchronized time scale. Recording started 10 min into injection of the CEC-P1 sample and was terminated after 37 min into recording since the injection buffer distort light scattering and refractive index data.

LS measurement on molecular weight from the SEC purification is found in table 5. The measured concentration after SEC purification was calculated to 24.9 µM of SPI with a total volume of 13.5 ml collected, by using Beer's law and spectrometer measurement at 280 nm.

Table 5. Molecular weights from peaks from LS-data. The calculated molecular weight was derived from LS data and the calculation was performed in ASTRA software. Concentration measurement is from pooled fraction from collected fraction during LS-measurement.

Peaks from SEC Chromatogram	Mw [kDa]	Absorbance at 280 nm
Peak 1	128.3 ± 1.3*	0.059
Peak 2	27.9 ± 0.25*	0.697

**Error is machine estimated and not statistical experimental derived.*

Result of light scattering to investigate interaction between SPI acting on pancreatic proteases

The experimental method showed a characteristic pattern for all samples. Namely three characteristic UV280 peaks followed by two peaks with refractive index and one peak for light scattering, see figure 16. Shows one of the experiments, but should be noted that all experiment had the same characteristics. Therefore, all data was not in the report.

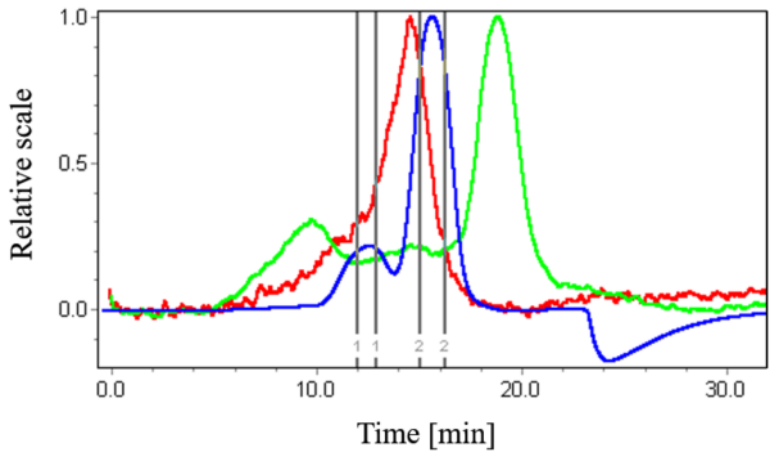

Figure 16. Light scatter plot generated by ASTRA software. Recording started 20 min after injection of sample into the LS system. The plot shows the result after injecting 50:1 inhibitor to enzyme ratio sample of 1 ml, which have been separated by an isocratic chromatography using a Superdex column. The data shows green which is absorbance at 280 nm, red is light scattering data and blue is change in refractive index, the y-axis is a relative scale to fit all data in same plot. Grey enclosure defines peaks which will be used to calculate the M_w.

Peaks was defined after refractive index, this was to produce the M_w by letting ASTRA software preforming the calculation in page 13. The resulting M_w for experiment is shown in table 6, which both defined peaks from LS-data, see figure 16. It should be noted that some experiment from the method was not preformed due to lack of SPI sample.

Table 6. Resulted molecular weights from calculation performed by ASTRA software of trypsin and inhibitor solution.

Experiment Inhibitor:enzyme ratio	Molar weight peak 1 [kDa]	Molar weight peak 2 [kDa]
50:1, trypsin	105.6 ± 2.1 kDa*	26.5 ± 0.5 kDa*
50:1, Chymotrypsin	97.4 ± 0.8 kDa*	21.9 ± 0.2 kDa*

**Error is machine estimated and not statistical experimental derived.*

Result from kinetic measurements in the non-conjugated state

The initial absorbance data from product formation at 410 nm, were first converted to enzymatic unit's U which is µM/min. The initial rate for each experiment was calculated by first adjusted to zero U, then using linear regression in MATLAB, the slope is the initial rate v_0, this was done for both the systems containing trypsin and chymotrypsin. To calculate interesting enzymatic property for trypsin and chymotrypsin interacting with and without SPI a saturation curve was created, were initial rate was plotted against concentration, see figure 17. All saturation curves were created in GraphPad Prism 7.01, the program also derives V_{max}, K_m and K_i numbers under the assumption that the inhibitor is competitive, see table 7, also for high inhibitor concentration was not included in deriving parameters as it did not fit regression model properly. It should be noted that trypsin result showed a large degree of variation between experimental sets. This was most likely do to physiological changes in the cuvette during measurement, due to BAPA stock improperly dissolved.

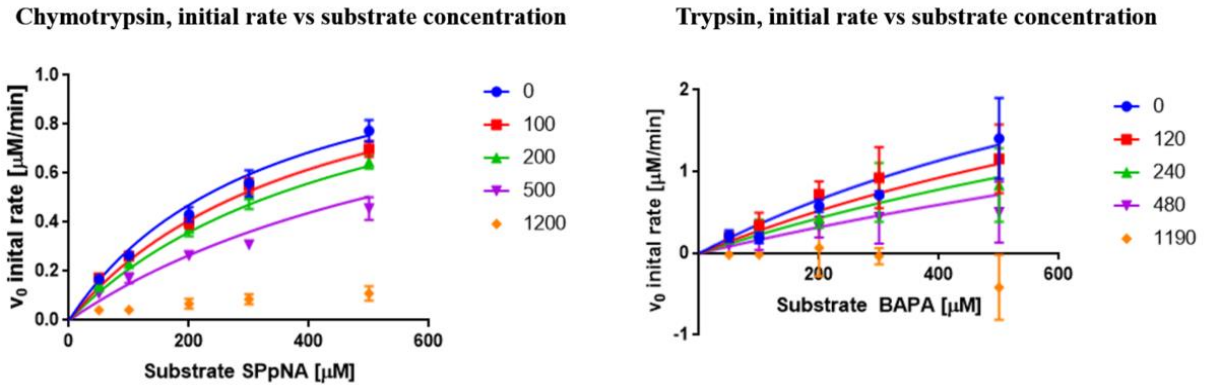

Figure 17. Saturation curve with initial velocity vs substrate concentration for each experiment with different inhibitor concentration, the legend for each inhibitor concentration is in nM. The data points are represented by the points in the graph, while the line represents the non-linear regression for each saturation curve. Note that for high concentration of inhibitor (1200 nM chymotrypsin and 1190 nM trypsin) the remaining activity was too low to be processed by the regression software for competition inhibition in Graphpad Prism 7 software. Therefore, the regression was not included in the model, but data points are included in the graph.

Table 7. Catalytic activity result from measuring kinetics in cuvette with enzyme and SPI in the non-conjugated state.

Enzyme	V_{max} [µM/min]	K_m [mM]	K_i [mM]	K_{cat} [s^{-1}]	R^2
Trypsin	4.2 ± 2.5	1.1 ± 0.9	0.38 ± 0.14	0.035 ± 0.020	0.67
Chymotrypsin	1.3 ± 0.1	0.34 ± 0.04	0.40 ± 0.05	0.01 ± 0.0008	0.96

Values for V_{max}, K_m, K_i and R^2 is derived from Graphpad Prism 7 and data for 1200 nM inhibitor is not included in the parameter calculations.

Result from immobilization onto aluminium oxide membrane

After immobilizing enzymes and SPI inhibitor onto the aluminium oxide the amount of substance that might have been absorbed was estimated by measuring absorbance at 280 nm of the reaction mixture. A subtractive algorithm was employed, where the amount immobilized was calculated from the difference in absorbance of the reaction sample between the start and end of reaction. Using the known extinction coefficient, the data were finally converted to molar amounts, see table 8.

Table 8. Estimated protein sample absorbed onto the aluminium oxide membrane. The substance absorbed onto enzyme and inhibitor solution was calculated by absorbance at 280 nm and the loss of content are after 24 h.

Immobilized protein	Initial substance in reaction mixture [nmol]	After incubation substance in reaction mixture [nmol]	Number of AlOx membranes [nr]	Absorbed in membranes [nmol] ([%])
Trypsin	100	7.1	10	93* (93 %)
α-chymotrypsin	100	25.0	10	75* (75 %)
PSPI-P1	15.5	11.9	5	3.6 (23 %)

**It should be noted that it is an uncertainty about the total protein content immobilized, due to that the wash solution was not collected after immobilization and measured amount of protein from the wash steps.*

Result from Experiment 1

Interaction with free enzyme and conjugated SPI is detected by change in retention time between conjugated SPI and non-conjugated AlOx membranes, as described under page 16. Running a series with three different concentrations of chymotrypsin solutions, namely 10, 15 and 30 µM, through the conjugated and non-conjugated AlOx membranes. During each run data was collected for 3000 seconds detecting the absorbance at 280 nm of the membranes flow through, see results at figure 18. The result clearly indicated that the lower concentration of chymotrypsin had a delayed peak with 200 s, it should be noted that the blank AlOx membrane did not have any immobilized APTS spacer on the surface.

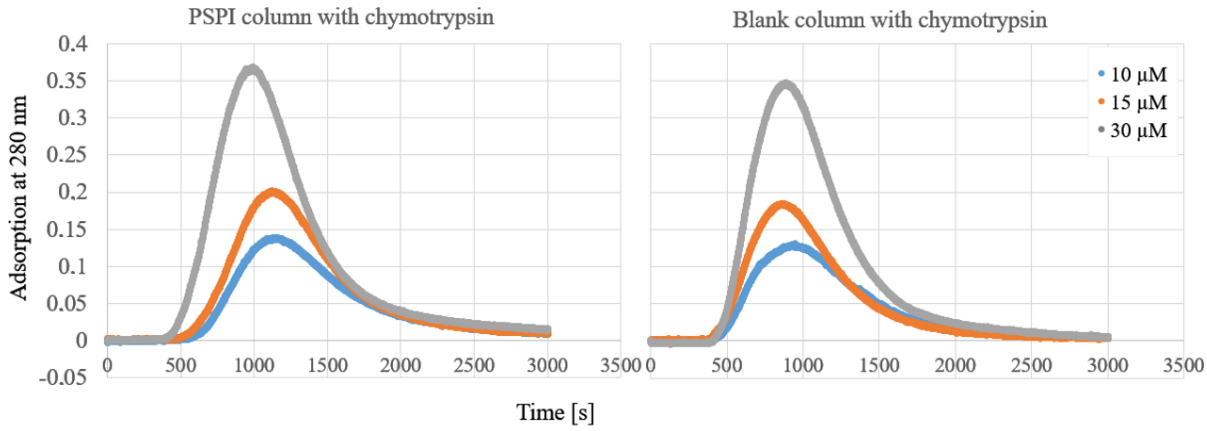

Figure 18. Graph of chymotrypsin elution time in the SPI column compared with the blank column.

To further indicate that delay was due to inhibitor-enzyme interaction, reference molecules which should not interact with the inhibitors was tested. The references serum albumin and myoglobin were used as they most likely do not interact with the inhibitors and are common reference molecules used. The result is shown in figure 19. In this experiment the delay is not really clear as trypsin and chymotrypsin have the same elution time as myoglobin, while serum albumin have an earlier elution time with about 80 s, the result for 10 µM chymotrypsin that did not show any peak is not known.

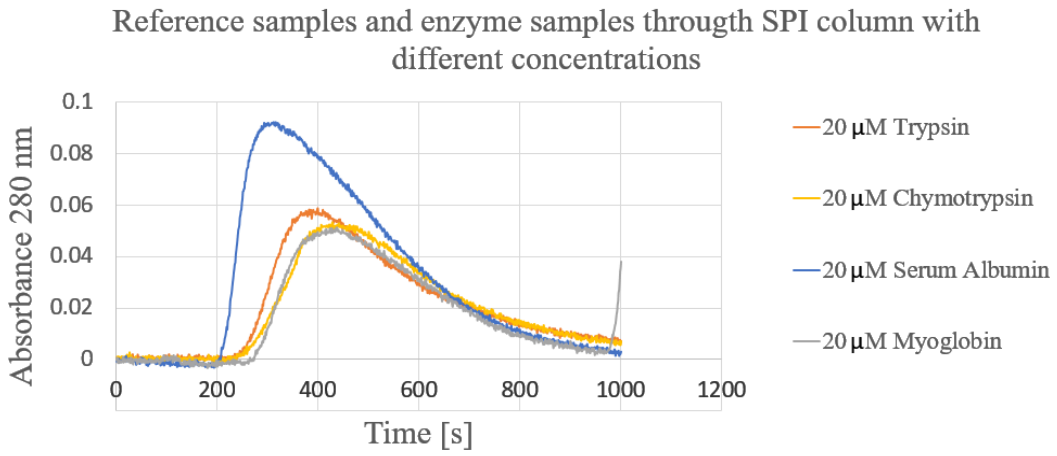

Figure 19. Continuous measurements of the absorbance at 280 nm of the flow through, from the membrane holder containing AlOx membrane conjugated with SPI, for 1000 seconds after sample injection. The Sample injected was 20 µM sample solution containing either trypsin, chymotrypsin, serum albumin and myoglobin, which is represented by different colours in the graph, see legend on the right side.

Result from Experiment 2

As the experiment to measure catalytic activity for trypsin and circulating BAPA mixed with SPI was not completed fully, it should be seen as a preliminary result. The biggest flaws were that the result is based on one experimental series and not a triplicate series, due to lack of time. Also several problems have made it problematic to get good results, which will be discussed in the conclusion. The experimental run can be seen in figure 20.

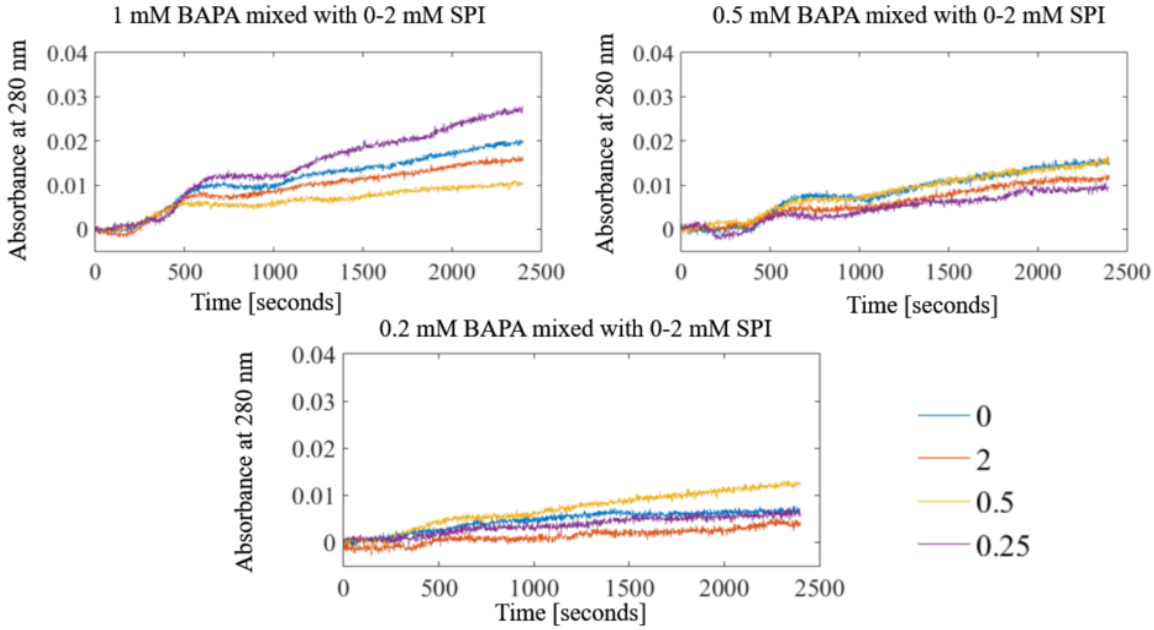

Figure 20. Circulating different BAPA and SPI concentration through a trypsin column and simultaneously measuring absorbance at 410 nm in flow cell. The plots represents absorbance vs time of BAPA and SPI solution circulating in the system overtime. For each BAPA concentration of 1 mM, 0.5 mM and 0.2 mM a series of inhibitor concentrations were used, which is described in the legend. Blue includes no inhibitor, purple contains 0.25 µM of inhibitor, yellow contains 0.5 µM of inhibitor and orange contains 2 µM of inhibitor.

From the initial dataset, the time course of absorbance from 2000-2400 sec was collected and converted to substance change rate in µmol. The selected dataset was then used to do linear regression and reveal the initial rate (µmol/min). The 2000-2400 sec interval was chosen since the system was expected to be stabilized at that time with respect to initial dilution, see figure 21.

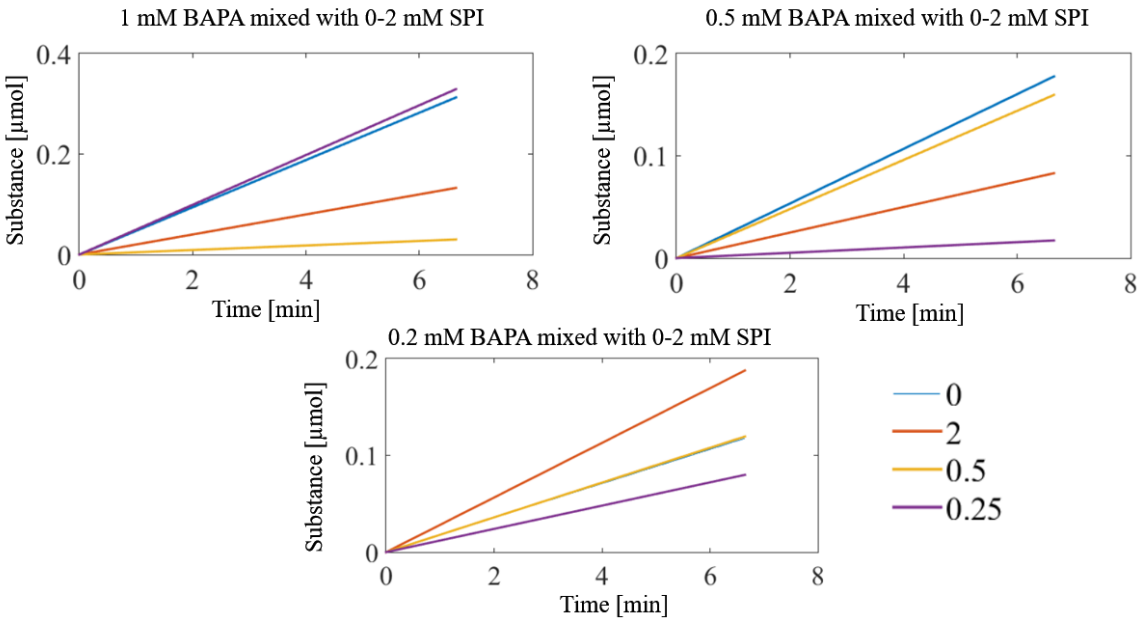

Figure 21. Linear regression from 2000-2400 sec interval from original data. The slope is in activity units U, by plotting substance in µmol vs time in min. The plots are different BAPA concentration, while the datasets are different inhibitor concentration which can be seen in the legend. Blue includes no inhibitor, purple contains 0.25 µM of inhibitor, yellow contains 0.5 µM of inhibitor and orange contains 2 µM of inhibitor.

Conclusion

Purification

Purification of the two homologous SPI was most likely successful by the parallel purification method described in this report. The inclination that indicates that the purification was successful is the fact that we have two peaks that show inhibitory activity against the pancreatic proteases trypsin and chymotrypsin.

It should also be noted that this method is an affordable and scalable approach to achieve high purity of the SPI target. Most of the method is reasonable for upscaling the process, except the homogenization strategy that requires something more powerful than a kitchen mixer. The crude extraction could be optimized or should be adjusted for large scale production. The reason why the crude extraction method was chosen was due to earlier reports and that it is a convenient method for laboratory scale. Also, method optimization for the upscaling was not a scope for this project.

Characterization

Concentration determination

The concentration determination consisted of using beers law which uses a protein specific extinction coefficient to calculate the protein concentration on the linear region. As there are two different homologous SPI inhibitors, with a small difference in extinction coefficient, is was hard to determine the exact homology which was in the different samples. Therefore, a mean value of the two extinction coefficients was used, which seems as a reasonable approximation of the concentration as it is only a small difference between the coefficients. The total protein content of purified PSI was calculated to 6.9 mg for CEC-P1 and 2.2 mg of CEC-P2 with concentrations of 24.9 µM and 11.4 µM, respectively.

SDS-page

The SDS-page from the CEC separation shows three components for peak from CEC-P1 and from CEC-P2 two components are distinguished. It should be noted that the double band between 30 kDa and 20 kDa probably are representing one protein, but with artifacts from the sample preparation, possibly incomplete unfolding, which makes some protein to be in an unfolded and some protein in the folded state. From the SEC step, only one peak remains present which shows inhibitory activity against SP and that fraction shows one band in the SDS in the 20-30 kDa area. This protein is most likely the SPI-1 or SPI-2 homologous protein discussed in a previous thesis project (Eriksson 2016).

Kinetic measurement and inhibitory action analysis

Light scattering analysis

Light scattering shows indication of aggregation in the first RI peak, which is about four times the M_w of the second RI peak. The characteristic formation with two large peaks, with a small peak in between the large peaks in the UV280 data was seen in all experiment. It can be mentioned that this was also seen for the pancreas SP elastase, but as this report focuses on trypsin and α-chymotrypsin the result was not mentioned in the report. The result is interesting because it further indicates the multiple-to-one interaction with enzyme and inhibitor, which have been suggested by previous author (Meulenbroek *et al.* 2012). An interesting fact is that PSPI might form a four molecule complex when heated, which also has been observed by other research groups (Pouvreau *et al.* 2005). Therefore, the result might be this aggregate form and not the enzyme-inhibitor complex which was desired.

Non-conjugated activity measurement

The kinetic result from non-conjugated enzyme-inhibitor interaction in quartz cuvette was moderate, where the kinetic measurement for chymotrypsin and SFpNA was clear in showing the inhibitory action of SPI and chymotrypsin. For the activity measurement with BAPA and trypsin the result had some problem which effected the calculation of the Km and Ki, but the inhibitory action of SPI on SP was still very clear. The problem with the measurement was that BAPA did not dissolve in the stock solution properly giving a dilution effect when measuring activity. This was proven by measuring at 490 nm, where BAPA was mixed with buffer without enzyme. The observation was a drop in absorbance, which probably was caused by physiological changes in the solution, like a change in turbidity when diluting BAPA. The kinetic parameters could not be derived from the highest inhibitor concentrations, for both trypsin and chymotrypsin, due to bad regressions based on a competitive inhibitor model. The reason for bad model fitting is still unknown, but it is most likely due to the very low residual activity which can no longer be processed by the GraphPad Prism 7 regression software. From a qualitative point of view, these data are in accordance with the other data sets.

Conjugated activity measurement

The experimental method for trying to measure catalytic activity on the conjugate gave no information about the kinetics. There are several reasons why the experimental setups did not achieve any immediate result. The main reason why there are some challenges to this method is first of all because the number of immobilized protein is low and the contact time is short for the experimental method presented. Therefore, the product formation is very low and thus the response is very weak. A solution to this problem is slowing down flow rate and increasing number of membranes. Another problem for the circulation experiment was that dead volume was too high and thus dilution, stabilizing and diluting sample too much for the given samples, basically a teabag in the sea.

A lot of practical problem also slowed down the process during this project like leaking pipes due to high pressure most likely being created by the membranes. Some problems were also due to air bubbles forming in the top and below chamber of the membranes which disturbed the measurement and also the flow rate changed by 30 % for experiment where catalytic activity for trypsin and α-chymotrypsin was measured after passing the bioreactor with conjugated serine protease inhibitor.

The conclusion working with this method is that they need more work before any comparison between the non-conjugated experiment data, which was the main goal with this project. No conclusion can therefore be reached from the methods in this project. But it is not clear if the methods can reach any valuable result if the method is improved significantly.

Ethics and society

As society have progress due to technology development as the internet and the personal computer science does not only exist in the university corridor anymore. Therefore, it is important to discuss about the impact of research conducted today. There are obviously no ethical aspects working with potato, neither in the case of GMO (genetically modified organisms) nor using up the potato, since the target proteins are pre-existing in the potato tuber. Also resource aspects should be considered as the biomaterial is a food product. SPI extraction should not compete with other necessities of the potato and the PSPI might have commercial uses and need to be extracted for large scale production. This is probably not a big problem as PSPI could be extracted as a side product from potato starch production. Therefore, it can quite naturally be applied for largescale production without using any extra resources. Also, in the unlikely case that potato would become a shortage, there are other species that have very similar Kunitz-type SPI, like soybean. There are in general only good aspects with this research as SPI could have medical application or application in food treatment and enzyme technology.

Further work

The characterization of the serine protease inhibitor that was purified needs to be verified by either "finger printing" analysis with mass spectrometry or amino acid analysis, to ensure that SPI-1 and SPI-2 protein characterized (Eriksson 2016) is the same protein as in this thesis project. Also, AlOx membrane which have immobilized SPI protein should be sequenced using amino acid analysis. This method would break up the AlOx membrane and peptides bond to be completely sequenced. This experiment is important to consider as they prove conjugation of the SPI protein onto the AlOx and that the right protein was purified.

The end-point methods described in this report to measure activity of conjugated enzyme-inhibitor interaction should still be considered and tested more until an objective answer can be given.

It should be recognized that the activity measurement with conjugating measurement with current method described in this report have some problem, as it does not measure activity in real time; but instead is an end-point method. Another more reliant technique that measure interaction in real time is surface plasmon resonance (SPR) analysis, which is a robust technique to measure protein-protein interaction. SPR also requires that one of the two components must be immobilized onto dextran substrate coupled onto a gold surface.

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