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Purification and Technical Application of a Serine Protease Inhibitor from *Solanum tuberosum*

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Abstract

A candidate protein was partly purified from homogenized potato in two purification steps, including stepwise ammonium sulfate precipitation and cation exchange chromatography. The partly purified protein was tentatively identified by MS fingerprinting as a serine protease inhibitor annotated Serine Protease Inhibitor-2, with a molecular weight of 20.1 kDa. The purified inhibitor showed strong inhibition of trypsin and α-chymotrypsin for all purified fractions and measurable inhibition of elastase.

Keywords
Digestive enzymes, Serine protease inhibitors, Protein purification, Protein characterisation, Technical application, *Solanum tuberosum*

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Purification and Technical Application of a Serine Protease Inhibitor from *Solanum tuberosum*

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Populärvetenskaplig Sammanfattning

I människans matsmältningssystem finns en rad olika enzymer som bryter ned proteiner och hjälper till att smälta den mat vi äter. Hos friska människor är denna proteinnedbrytande aktivitet kopplad till matsmältningssystemet inuti kroppen och neutraliseras sålunda på vägen ut ur kroppen. Personer som lider av mag- och tarmsjukdomar kan få problem med de neutraliserande stegen av de proteinnedbrytande enzymerna och får därmed en hög enzymatisk aktivitet i sin avföring. Vid kontakt med avföring, exempelvis vid användandet av blöja, i samband med dessa komplikationer, kan skador på huden uppstå som följd av den förhöjda enzymatiska aktiviteten.

Det har visat sig finnas proteiner i vanlig potatis som kan inhibera dessa proteinnedbrytande enzymer. Ett sådant protein kan därmed hämma den proteinnedbrytande aktiviteten som kvarstår i avföring hos personer som lider av mag- tarmsjukdomar.

Detta examensarbete har i tre steg delvis renat fram ett protein ur potatis, en okontroversiell naturlig källa till målproteinet. Det renade proteinet visade sig ha inhiberande effekt mot essentiella proteinnedbrytande enzymer som finns i matsmältningssystemet, trypsin, α-kymotrypsin och elastas. Reningsmetodens alla steg har verifierats och innehåller proteinet av intresse och har kapacitet för att skalas upp.

Proteinet som delvis renats fram har med stor sannolikhet visats vara en kompetitiv inhibitor till serin proteasen namngiven: Serine Protease Inhibitor-2, med en molekylvikt av 20.1 kDa. Inhibitorn har visat sig inhibera tre olika enzymer, trypsin, α-chymotrypsin och elastas, vilka alla är viktiga proteinnedbrytande enzymer i människans matsmältningssystem. Proteinet har bedömts vara lämpligt för immobilisering, och vidare studier kan visa hur inhibitorn kan användas för tekniska applikationer.

Examensarbete 30 hp
Civilingenjörsprogrammet i Molekylär bioteknik

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List of Abbreviations

BAPA	N-α-benzoyl-DL-arginine-p-nitroanilide
BTEE	N-benzoyl-L-tyrosine-ethyl-ester
DMSO	Dimethyl Sulfoxide
DTT	Dithiotreitol
E-CR	Elastine Congo-Red
HIC	Hydrophobic Interaction Chromatography
IEX	Ion Exchange Chromatography
IMAC	Immobilized Metal-ion Affinity Chromatography
LS	Light Scattering
MS	Mass Spectrometry
SDS-PAGE	Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SPI-1	Serine Protease Inhibitor-1
SPI-2	Serine Protease Inhibitor-2

1 Introduction

The digestive system in living organisms and the enzymology behind it have been of both commercial and pharmaceutical interest in regard to the catalytic mechanism and the corresponding inhibitors. Proteins secreted from the pancreas e.g. enzymes that break down the dietary proteins are essential in the digestive system of humans [1]. The pancreatic enzymes in healthy humans are neutralized on the way out of the body. However, studies show that patients with bowel diseases, hence dysfunction in the digestive system still have high proteolytic activity in the human waste [2]. Skin injuries together with frequent contact with human waste are connected with dysfunctions and diseases as gastro intestinal bleeding, liver failure and Chron's disease.

It has been revealed that proteins extracted from *Solanum tuberosum*, potato, have inhibiting effects on proteolytic activity from human waste [2]. This suggest that treatment with inhibiting proteins may decrease skin injuries for persons with gastro intestinal diseases in frequent contact with human waste. This master thesis aims to develop purification protocols for and to characterise proteins from *Solanum tuberosum* with inhibiting effects on pancreatic enzymes found in the human digestive system.

1.1 Background

1.1.1 Enzymes and Catalytic Activity

Enzymes, i.e. proteins with specific catalytic functions, have various target molecules and specificity, which are driven both by the environment and by the structure of the active site i.e. the binding pocket of the enzymes [3]. Enzymes will enhance the conversion from substrate to product by decreasing the activation energy in the transition state of the specific reaction [4]. The transition state refers to the highest amount of potential energy to obtain the conversion. The decrease of energy is due to one or several intermediate states that the enzymes are creating. Generally the enzyme creates a reversible complex with the substrate and releases upon product formation. Hence, the enzyme is not consumed during the reaction, resulting in very high efficiency.

One important chemical reaction is the protein degradation in living organisms. The degradation of proteins is commonly known as proteolysis, i.e. hydrolysis of proteins [5]. The hydrolysis mechanism uses water molecules to break chemical bonds. Thus, proteolysis uses a water molecule to break a peptide bond (Figure 1).

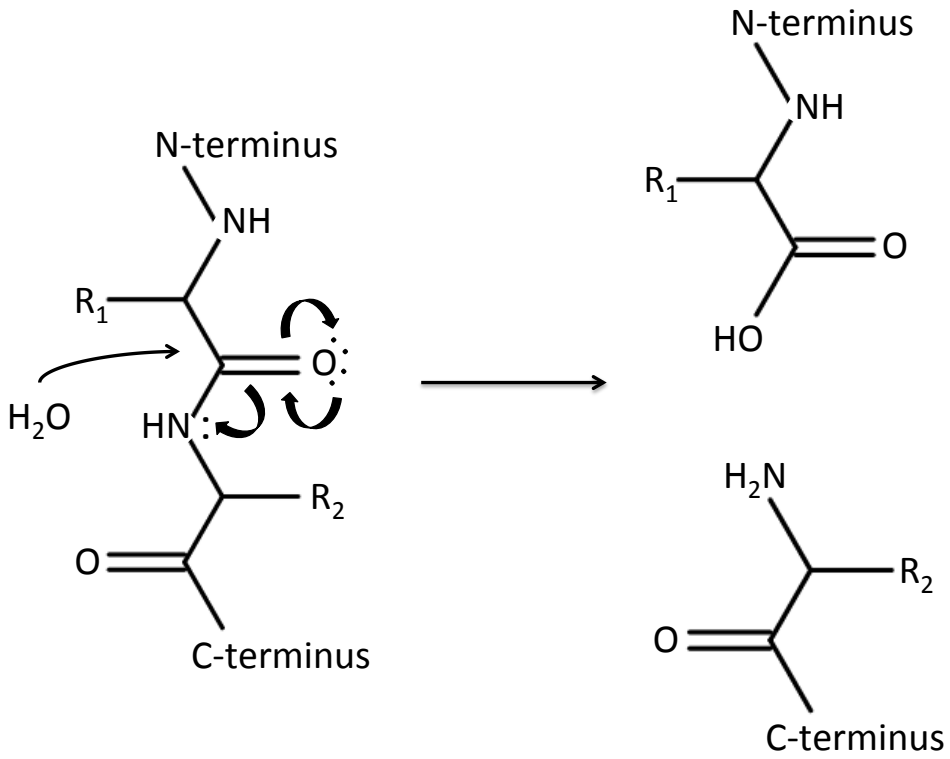

Figure 1: Schematic drawing of proteolysis. A water molecule performs a nucleophilic attack on the peptide bond resulting in two smaller peptides [5]. The drawing is based on the non-enzymatic hydrolysis of a peptide bond.

Spontaneous proteolysis occurs naturally but very slow, which for example has an important role in both archaeology and palaeontology. Proteins and other materials that are sustainable over time can give important historical guidelines due to the natural but very slow break down of proteins [5]. However, proteolysis, hence hydrolysis, requires proteases to obtain a faster reaction mechanism, enzymes that catalyse the proteolysis. Proteases can be found and have crucial roles in all living organisms e.g. humans, plants, bacteria, archaea and viruses [2,6-9]. The classification of proteases is due to the nucleophilic amino acid in the active site, commonly serine, cysteine, aspartic acid or a metal [10]. Serine proteases, in particular, have a catalytic triad in their active site, a specific amino acid motif of three amino acids; one nucleophile, one acid and one base that act together in the catalytic mechanism [11]. This present work will focus on serine proteases, where serine is the nucleophile residue in the catalytic triad.

1.1.2 Serine Proteases

Serine proteases are proteolytic enzymes where serine is the nucleophilic amino acids in the catalytic triad [2,10,12]. Histidine and aspartic acid are the two other amino acids in the catalytic triad of serine proteases (Figure 2). The general reaction mechanism for serine proteases is as follows; serine acts as a covalent catalyst, histidine acts as both an acid and a base catalyst and aspartic acid stabilizes histidine during the peptide bond breakage [13]. However, all serine proteases use the same catalytic triad and reaction mechanism while the specificity between different serine proteases is driven by different structures of the S1 pocket, a part of the active site [14].

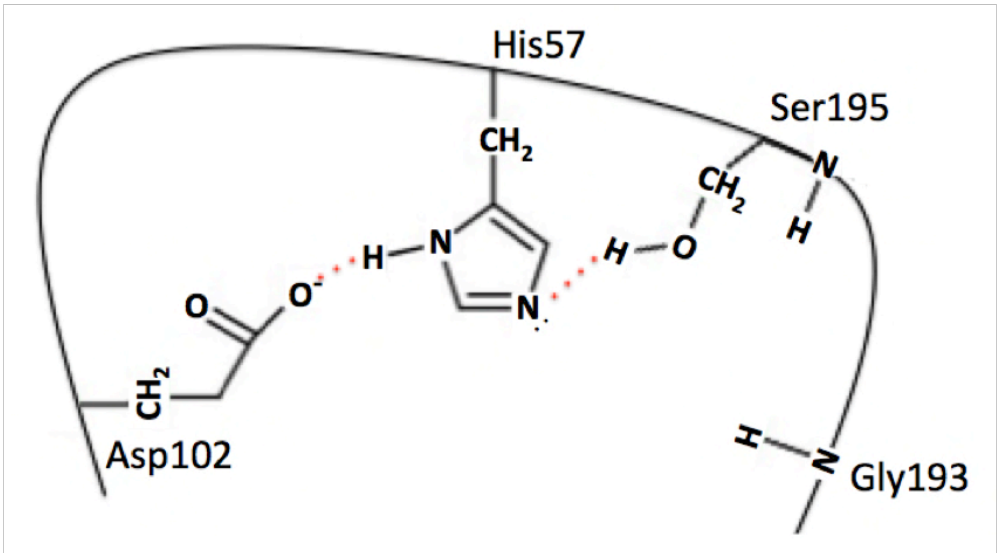

Figure 2: Schematic drawing of the active site of α-chymotrypsin. The catalytic triad of α–chymotrypsin consists of an aspartic acid amino acid numbered 102, interacting with histidine57, which in turn interacts with serine195. The drawing is based on the three-dimensional structure of α–chymotrypsin obtained by Matthews *et al*. [11].

The serine protease reaction mechanism behind the breakage of peptide bonds is divided in two steps, formation of a new amine end upon covalent catalyst and formation of a new carboxyl end upon nucleophilic attach of a water molecule (Figure 3) [13]. The release of the amine component bond will start with the forming of an unstable tetrahedral intermediate, i.e. the serine residue binds covalent to the carbon in the peptide bond and form a reactive enzyme-substrate complex. Following that, histidine donates a proton to the nitrogen in the peptide bond, hence acting in an acid-catalyst way, causing the peptide bond breakage, and thus release of a small peptide with a new amine terminus. This part of the mechanism acts in a non-specific manner, where this part of the peptide does not contain the serine amino acid, hence not the enzyme-specific target amino acid. In the second step, histidine instead works

as a base catalyst on a water molecule, accepting one proton, whereas the oxygen in water performs a nucleophilic attack on the carbon in the former peptide bond. A new unstable tetrahedral intermediate is created. When histidine donates back a proton to the serine residue the covalent bond breaks and a new carboxyl terminus and the second peptide has been formed. The enzyme is at the same time restored back to its original state.

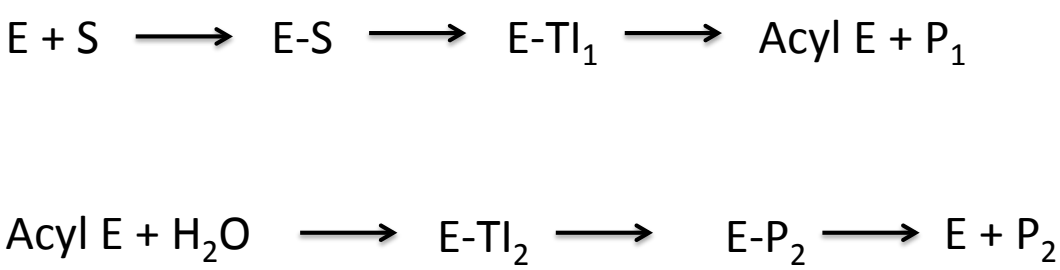

Figure 3: Summary view of the serine protease mechanism. Enzyme (E) and substrate (S) is forming an enzyme-substrate complex (E-S). The serine residue in the active site binds covalently to the carbon in the peptide bond [13], resulting in the first unstable tetrahedral intermediate ($E\text{-}TI_1$). The histidine amino acid acts as an acid catalyst and forms a new amine terminus to the first new smaller peptide (P_1) together with an acyl-enzyme intermediate (Acyl E). With the help of the acyl-enzyme intermediate, a water molecule performs a nucleophilic attack on the carbon in the former peptide bond, hence creating a new unstable tetrahedral intermediate ($E\text{-}TI_2$). Histidine then donates back a proton to the serine residue, which breaks the covalent bond of serine, creating the second new smaller peptide (P_2).

1.1.3 Pancreatic Serine Proteases

The glandular system in humans has endocrine and exocrine glands that secretes various substances [15,16]. Endocrine glands secrete directly into the bloodstream while exocrine glands secrete through ducts or tubes onto epithelial surfaces. One organ in humans that serves as both an endocrine and exocrine gland is the pancreas. The pancreas is located in the abdominal cavity, connected to and surrounded by the duodenum. The pancreas secretes essential hormones such as insulin and glucagon into the bloodstream as endocrine function [15]. In parallel to that, the pancreas secretes pancreatic juice via ducts to the digestive system as exocrine function [16].

Various enzymes are synthesised and stored in the pancreatic juice, among them serine proteases, in inactive forms, zymogens [17-19]. The pancreatic enzymes are stored inactive as a self-protection mechanism for the pancreas. Trypsin, chymotrypsin and elastase are three enzymes present in the pancreatic juice, which in their active form have essential roles in the human digestive system.

Zymogens from the pancreatic juice require activation to receive enzymatic activity [17-19]. Trypsinogen is secreted from the pancreas into the duodenum and cells in the duodenum produce enterokinase while trypsinogen is present [17]. Hence, trypsin is autolytically activated from trypsinogen in the presence of enterokinase in the duodenum. Upon activation trypsin works as a specific serine protease, having the catalytic triad and reaction mechanism mentioned before. Trypsin cleaves peptide bonds at the carboxyl end of the positively charged amino acid residues, i.e. lysine and arginine, which are two out of three amino acids that have positive side chain polarity. The specificity of trypsin is driven by an aspartate amino acid in the bottom of the S1 pocket, a specific pocket structure only found in trypsin serine protease [20,21].

However, trypsin activates chymotrypsinogen, another zymogen secreted from the pancreatic juice [18]. Chymotrypsinogen is activated to chymotrypsin, with the same catalytic triad and reaction mechanism as trypsin but with different selectivity according to the structure in the S1 pocket. Chymotrypsin cleaves at the carboxyl ends of amino acids as methionine, phenylalanine, tyrosine and tryptophan. Hence, at amino acids those are large, hydrophobic, and non-polar and fit into the relatively deep and hydrophobic S1 pocket of chymotrypsin [11,20].

Further, trypsin also activates elastase, a protein that catalyses the hydrolysis of elastin proteins from its zymogen [19]. Elastase also has the same catalytic triad and reaction mechanism as trypsin and chymotrypsin. But compared to the above mentioned, the selectivity is driven by two valine amino acids acting as a steric hinder in the S1 pocket [20,22]. As a consequence, elastase cleaves peptide bonds at the carboxyl end of glycine, valine, alanine, leucine and isoleucine, aliphatic amino acids, hence non-polar and that fit into the specific elastase S1 biding pocket.

Trypsin, chymotrypsin and elastase are thus pancreatic enzymes acting in the digestive system upon activation. In a healthy digestive system the proteolytic activity is neutralized on the way out of the body via adsorption and endogenous inhibitors in the distal ileum, followed by bacterial neutralisation in the large intestine [1,2]. However, dysfunctions and diseases where the proteolytic activity cannot be neutralized in the pathway may cause skin injuries, especially for persons in frequent contact with human waste e.g. patients with gastro intestinal bleeding, liver failure and Crohn's disease [2].

1.1.4 Enzyme Kinetics

The mechanism of a catalytic mechanism coupled to enzymes is described in enzyme kinetics. Generally, enzymes will speed up the specific reaction for a specific substrate [4];

hence the velocity of the reaction is directly dependent on the change in substrate concentration as described in the rate equation, Eq 1.1.

$$\frac{\Delta P}{\Delta t} = v = k\,[S] \qquad \text{(Eq 1.1)}$$

The product formation over time reveals the rate constant, k, which corresponds to the speed or efficiency of the specific reaction depending on the substrate concentration [S]. However, the velocity, v, is the slope of the particular progression curve obtained by the enzymatic reaction. The initial velocity, v_0, of the specific reaction corresponds to the linear proportion of the progression curve at a specific substrate concentration.

Enzyme-catalysed reactions are described mathematically according to the Michaelis-Menten equation, Eq 1.2 [23]. Here, the equation constants describe the specificity and efficiency of one particular enzyme, hence important properties for a specific enzyme-catalysed reaction. The equation is obtained from the initial rate, v_0, for different substrate concentrations, where the result is indicated of enzyme-substrate complex formation upon a hyperbolic curve i.e. simple dissociation.

$$v_0 = \frac{d[P]}{dt} = \frac{V_{max} * [S]}{K_m + [S]} \qquad \text{(Eq 1.2)}$$

In the Michaelis Menten equation, V_{max} refers to the maximal rate of the reaction at maximal substrate concentration. Further, K_m refers to the substrate concentration at which half of the enzymes' active sites are saturated [23]. The rate constant k_{cat} is determined at conditions where the substrate concentration in the sample is much greater than the substrate concentration at K_m. The rate constant k_{cat} refers to the turnover number for enzymes, and is calculated according to equation Eq 1.3 [24]. The ratio between the rate constants k_{cat} and K_m determines the catalytic efficiency of the enzyme active in the reaction. The ratio between k_{cat} and K_m is also used to compare the enzymatic effects to different substrates. The quantification of proteins in solution is required to estimate the specific activity from the enzymatic activity. Specific activity shows enzyme activity per mg protein in solution i.e. the specific activity reveals the purity of the enzyme in the protein solution.

$$v_{max} = k_{cat} * [E] \qquad \text{(Eq 1.3)}$$

1.1.5 Enzyme Inhibition

Enzyme inhibitors are molecules that can change or inhibit the enzymatic activity [25]. Enzyme inhibition is either reversible or irreversible, in regards to the strength and the character of the binding to the enzyme. Reversible inhibition is generally based on non-covalent interactions between the enzyme and inhibitor in three different ways: competitive inhibition, non-competitive inhibition and uncompetitive inhibition. The inhibitor can easily dissociate from the enzyme for all reversible pathways.

A competitive inhibitor will bind with a non-covalent interaction to the enzyme and in some way block the active site of the enzyme. The enzyme can therefor not bind to the substrate and it will decrease the enzymatic activity. The enzymatic activity will be dependent on the balance between the substrate and inhibitor concentration. By increasing the substrate concentration the substrate can outcompete the inhibitor. The maximum velocity of the reaction with this type of inhibition will be held constant, the apparent Michaelis constant will increase, and the affinity of the enzyme to the substrate will be lowered.

In the case of non-competitive inhibition, on the other hand, the inhibitor will not bind to the active sites; it will bind to another site of the enzyme. This leads to inhibition regardless if the substrate is bound to the active site or not. A three-dimensional structural change of the enzyme will decrease the enzymatic activity. This type of interaction will reduce the maximum velocity of the reaction.

In a third alternative, namely uncompetitive inhibition, the inhibitor will only bind to the enzyme when the substrate is bound to the active site. This inhibiting process will both decrease the maximum velocity of the enzyme and the Michaelis constant.

However, mixed inhibition is a combination of competitive and uncompetitive inhibition, the inhibitor can bind to both free enzyme and to a complex of enzyme already bound to the substrate. The inhibitor will bind to the active site or to another site of the enzyme depending on the affinity of the two states. In this case the kinetics either decrease or increase the apparent Michaelis constant in regards to the state of inhibition. However, the maximum velocity for mixed inhibition will be decreased.

Irreversible inhibition uses strong interactions between the enzyme and inhibitor, thus covalent or non-covalent bindings, where the inhibitor does not easily release from the enzyme inhibitor complex upon inhibition [26]. The inhibitor will bind to the enzyme so that the enzyme cannot bind to the substrate. The irreversible inhibition will decrease the enzyme concentration; hence decrease the maximum velocity of the reaction.

The kinetics of enzyme inhibition describes the regulation of the reaction mechanism for the specific enzyme. This mechanism is described by an inhibition constant, K_i [27]. As

mentioned before, inhibitors act as reversible or as irreversible inhibitors, where the reversible inhibition is competitive, uncompetitive or non-competitive. The experimental determination of the inhibitor effect, the kinetic behaviour of the enzyme, can be described mathematically by an apparent rate constant in presence of the inhibitor as in equation Eq 1.4.

$$V_0 = \frac{d[P]}{dt} = \frac{V_{max}*[S]}{K_{m,app}+[S]} \qquad \text{(Eq 1.4)}$$

To find the inhibitor constant K_i for the particular inhibitor the apparent $K_{m,app}$ is evaluated for the competitive inhibitor according to equation Eq 1.5, with regards to the concentration of the inhibitor used in the enzymatic assay.

$$K_{m,app} = K_m(1 + \frac{[I]}{K_i}) \qquad \text{(Eq 1.5)}$$

Inhibitors that inhibit the proteolytic activity are commonly proteins called protease inhibitors. Protease inhibitors has been found in seeds and tubers in various plant families, among them *Solanaceae*, potato family [2,28-30]. Proteases inhibitors extracted from *Solanum tuberosum,* potato, have shown inhibiting effects on digestive enzymes [2,28-30]. An earlier study [2] show that protein fractions from potato show inhibiting effects on pancreatic proteases such as trypsin, chymotrypsin and elastase. This suggests that protease inhibitors extracted from potato potentially can decrease the peri-anal skin injuries in individuals suffering from dysfunctions or diseases in the gastric intestinal tracts and have frequent contact with human waste.

1.1.6 Chromatographic Methods

Generally in biochemistry, protein purification is performed in series of separation techniques. There are various separation techniques available to purify proteins from a complex sample [31-36], for example a tissue extract contains a broad range of different proteins that can be separated form each other. Classical column chromatography techniques separate proteins according to their properties i.e. charge, affinity, hydrophobicity or size. The purification series are generally chosen in regards to the purpose of the procedure, analytical or commercial scales and overall yield together with time and cost considerations.

Ion exchange chromatography (IEX) separates proteins in regard to charged groups on the protein surface, arising from specific conditions in the environment [31]. IEX separation

relies on the reversible adsorption to oppositely charged immobilized groups in the stationary phase. IEX strategies are classified as cation or anion exchange chromatography depending on the charge of the sample components. However, a cation exchange chromatography has immobilized negatively charged groups to the stationary phase, hence positively charged molecules in the mobile phase will interact to the negatively charged stationary phase. Vice versa for anion exchange chromatography. The charge of the protein surface will be dependent on the pH in the environment and by selecting correct environment in the mobile phase, especially conditions for pH and salt concentration, the particular molecule of interest can be separated from a complex mixture of molecules according to the properties of the molecules. The separation of molecules will be dependent by three factors: the magnitude of the charge, the charge density and finally the concentration of the competing charged ions. However, these three factors will determine the selectivity of the separation in the ion exchange chromatography, and controlling the environment for the experimental set up can optimize the result. To purify a protein from a complex sample using IEX the correct resin need to be chosen together with optimal start conditions that allow unwanted proteins to pass through the column, and the protein of interest to bind to the column. The elution conditions also need to be optimal to elute the protein of interest in a small volume of the appropriate buffer.

Size exclusion chromatography (SEC) separates molecules in a sample mixture according to their molecular weight, i.e. their size [32]. The stationary phase for this separation method contains beads with pores of known size. With a buffer flow through the column the separation is due to the time spent in the column. However, the large molecules are not able to enter the beads, which lead to a limited volume inside the column for the large molecules, i.e. the space between the beads, the void volume of the column. This will result in with large molecules eluting in a smaller elution volume in comparison to the smaller molecules of the sample. Small molecules on the other hand are delayed in the column during the separation. The small molecules can enter the pores of the beads, and therefore have access to a larger volume inside the column and elute later than larger molecules. A sample mixture can be separated according to group separation or fractionation. Group separation will separate molecules in large or small molecules, useful when for example removing salts or low molecular weight impurities from a protein sample. Fractionation on the other hand will separate a mixture of molecules according to their molecular weight in a sample with high resolution. This method requires that the fractionation range of the resin include the molecular weight of all the molecules in the sample for optimized separation. The type of separation wanted and the exclusion limit of the resin; i.e. the smallest molecular mass that can diffuse into the beads are considerations required for optimising size exclusion chromatography.

The chromatographic experiments in this master thesis will be based on IEX and SEC. For future prospects, other separation techniques are available, for example affinity chromatography (AC) and hydrophobic interaction chromatography (HIC) [33-36]. Affinity chromatography separates proteins in regard to a specific ligand immobilized to the stationary phase, where Immobilized Metal Ion Affinity Chromatography (IMAC) is commonly used [34]. AC together with IMAC separates molecules according to their affinity to the ligands in the stationary phase and can be optimized by recombinant DNA technology. Recombinant DNA technology can be used to tag a specific protein with histidine residues for protein purification. However, recombinant his-tagged proteins and the technology behind it is not applicable for the purpose of this master thesis. As for HIC, biomolecules will be separated according to hydrophobicity [35]. Protein separation relies on the reversible adsorption to hydrophobic interactions in the stationary phase in high salt concentration. Often phenyl, octyl and butyl are hydrophobic groups immobilized to the stationary phase [36]. Generally, purifying proteins using HIC relies on the three-dimensional structure of proteins, i.e. on hydrophilic and hydrophobic patches on the surface of the protein.

Apart from SEC, all chromatography methods mentioned are based on the reversible adsorption to the stationary phase [31-36]. However the proteins have different properties in regard to specific conditions in the environment, meaning that environmental considerations are necessary for a proper purification procedure, hence considerations regarding ionic strength, pH and temperature.

1.1.7 Analytical Methods

Generally in biochemistry, qualitative and quantitative information from biological samples are obtained from analytical methods. Qualitatively methods determine if the substance of interest is present in the sample and quantitatively methods determine the amount of the substance of interest.

1.1.7.1 Protein Identification

Gel electrophoresis is an analytical method to analyse and separate proteins according to size, i.e. qualitatively determine if the protein of interest is present in the sample, as well as determine the purity of the sample [37]. Molecules in a sample are migrating through a gel matrix by the force from an electrical field. The matrix most commonly used is agarose or polyacrylamide, where the composition of the matrix determines the resolution of the separation. The gel electrophoresis is performed under two conditions: native (non-

denatured) or denatured. Native conditions analyse the natural structure of the analyte, hence the electrical force will be dependent on the over all structure. Further denature conditions will break the three-dimensional structure of the analyte and create one linear size analyte. The electrical force will then only depend on the mass to charge ratio of the analyte.

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a denaturing, polyacrylamide-based gel electrophoresis method to determine the size and purity of a protein mixture [38]. SDS is used as denaturing detergent to break the protein into a single polypeptide chain. The detergent contributes with an equally distributed negative charge along the polypeptide-backbone, ending up with mobility through the gel as a reproducible function of the mass of the polypeptides.

1.1.7.2 Protein Characterisation

As mentioned before, enzymes act as catalysts, which is one important property for protein characterisation. The catalytic mechanism and the specificity of one particular enzyme can be described by the relationship of product formation or amount of substrate used over time, in presence of the particular enzyme, i.e. by enzyme kinetics. The enzyme kinetics is described mathematically by the equations mentioned before. Worth to mention is that one particular enzyme acts with high specificity to one or a few selected substrates. In this master thesis the Michelis Menten constant K_m, the turnover number k_{cat}, the maximum velocity V_{max} and the inhibitor constant K_i will be characterised for three different enzymes; trypsin, α-chymotrypsin and elastase.

The characterisation of the enzyme kinetics is here measured spectrophotometrically by the hydrolysis of substrate in presence of enzyme, i.e. the enzymatic activity is measured as absorbance difference over time [39]. The enzymatic activity to the digestive enzyme trypsin can be measured by the cleavage of a synthetic substrate N-α-benzoyl-DL-arginine-p-nitroanilide (BAPA) that has a molecular weight of 435 Da. BAPA releases the coloured para-nitroaniline upon hydrolysis, in presence of trypsin, which can be measured spectrophotometrically at 410 nm. However, the function of substrate consumption or product formation over time, together with saturation curves for the specific enzyme to various concentrations of substrate, will give kinetic parameters of the reaction according to the Michaelis Menten equation [23].

Further, the enzymatic activity of α-chymotrypsin is measured by the same means as for trypsin and BAPA but in presence of the synthetic substrate N-Benzoyl-L-tyrosine ethyl ester (BTEE) that has a molecular weight of 313 Da. BTEE is hydrolysed by α-chymotrypsin and the substrate consumption; hence product formation can be measured spectrophotometrically

at 256 nm [40]. Finally, elastase hydrolyses Elastine-Congo Red (E-CR), with a molecular weight of 33 kDa, a synthetic substrate where the product formation can be measured spectrophotometrically at 495 nm [41].

The functional mass of components in a solution can provide important information about the molecular behaviour e.g. oligomer or complex formation. Light Scattering (LS) is an analytical method that provides this information [42]. This however can be translated into the average size of the particle over time; hence it can be used to determine the size and the molecular weight of a protein in a sample. In this master thesis the average mass of particles in a sample is measured by at setup that combines light scattering, differential refractive index and UV-signal.

Mass Spectrometry (MS) can be used for quantification of a complex sample. MS is a method with high precision to determine the exact mass for individual components of a sample [43]. MS uses the mass-to-charge ratio of gaseous ions to identify amount and compounds in a sample. The ion conversion regards to soft or hard ionization, corresponding to the degree of fragmentation of the sample. The mass analyser sorts the ions into a mass-to-charge ratio. Various techniques for mass analysers are available; the detection of ions measuring the charge induced or the current produced when ions hits the detector surface. These three main steps result in an experimental mass spectrum for the sample analysed, as quantification of the sample.

The protein concentration of a sample is of great interest for characteristics of a protein. Beer's law, Eq 1.6, can be used for converting the absorbance for a sample at a particular wavelength into a molar concentration of the same mixture [44]. The absorbance at 280 nm can be measured spectrophotometrically due to the aromatic rings of the amino acids tyrosine and tryptophan.

$$A = \varepsilon * c * l \qquad \text{(Eq 1.6)}$$

In Eq 1.6, A refers to the absorbance at a particular wavelength, ε is the molecular extinction coefficient at the same wavelength and l corresponds to the path length of the measurement. The equation can be used to approximate the protein concentration, when working in the linear range limit of Beer's law. However, this conversion requires a pure protein sample for obtaining the accurate concentration of the protein of interest.

The crystallization can verify the purity of the sample and the molecular structure of the protein as a last step of the analytical procedure of the protein purification protocol [45]. Crystallization is obtained by super saturation of the compound of interest. This is reached by

a phase transformation with a negative value of Gibbs free energy, ΔG_{tr}. The crystals are stabilises by minimizing the enthalpy ΔH_{tr}, which will determine the interactions of the molecules in the crystal. The super saturation is generating by evaporation, adding anti-solvent or by a pH shift. Crystallisation is used to determine the three-dimensional structure of a molecule and can also be used as an additional purification step, or for storage.

1.2 Aims and Objectives

The aim of this master thesis was to develop efficient and easily scalable protocols for the purification and characterisation of protease inhibitors from *S. tuberosum*. The protocols for the purification and characterisation were based on classical chromatography principles for preparation, combined with analytical characterisation methods such as electrophoretic purity analyses, physical characterisation by mass spectroscopy and specific inhibition assays.

This project did not need to consider ethical stance regard to the uncontroversial, natural and non-hazardous potato. The aim of this master thesis was reached and the work could be perform under correct ethical basics where no special privacy, integrity or conflict of interest needed to be considered to the subject during the present work. However, the technical applications for this project were of great interest due to the technical applications using an uncontroversial natural protein, with a process that has a low impact on the environment.

2 Materials and Methods

2.1 Instruments, Chemicals and Proteins

Centrifugation was performed in Allegra™ 25R Centrifuge, Beckman Coulter™ and spectrophotometric measurements were performed in a Shimadzu UV-1610 UV-visible spectrophotometer, Lambda Instruments. The ion exchange chromatographic experiments were performed on ÄKTA EXPLORER10, GE Healthcare with UNICORN software.

The light scattering was performed using a DAWN®EOS™ Enhanced Optical System supplemented with Optilab DSP Interferometric Refractometer, LKB Bromma, Monitor UV-M, Pharmacia and a 2150 HPLC Pump. The measurements were evaluated in ASTRA software.

Desalting columns PD10 and Superose 12™ gel chromatography column were provided from GE Healthcare. PhastGel 20% Homogenous and SDS-PAGE buffer strips were also provided from GE Healthcare. The Polypeptide SDS-PAGE Standard was provided from BioRad Laboratories. Chemicals for buffer preparation were of analytical grade. The enzymatic assay's synthetic substrates BAPA, BTEE and E-CR were obtained from Sigma Aldrich. Enzymes used in the assay were also obtained from Sigma Aldrich.

BabyBio S and BabyBio DEAE columns and WorkBeads ACT resin were a gift from Bio-Works AB, Uppsala, Sweden.

2.2 Experimental Procedure

2.2.1 Protein Extraction

3.89 kg King Edward potato, *Solanum tuberosum,* was peeled, cut and mixed to obtain a homogenous mixture. Coarse filtration was performed on the mixture and 0.2 % (w/v) ascorbic acid was added to the filtrate. The filtrate was centrifuged for 60 minutes at 5000 rpm in 4°C. The supernatant was collected and heated in water bath at 65°C for 15 minutes followed by 30 minutes of centrifugation at 5000 rpm at 4°C. The resulting supernatant was filtrated through filter paper. This filtrate was referred to as fraction *"Potato Crude"*. A 40% ammonium sulfate precipitation was performed on the *"Potato Crude"* fraction. The precipitate was collected by 30 minutes of centrifugation at 5000 rpm at 4°C and dissolved in a minimal amount of distilled water. The resulting solution was desalted in a PD-10 column equilibrated with 100 mM phosphate buffer pH 6.9, using gravity flow. This fraction was referred to as *"40% Potato Precipitate"*.

2.2.2 Chromatographic Methods

2.2.2.1 Cation Exchange Chromatography

25 mL of the*"40% Potato Precipitate"* fraction was applied onto a 5 mL BabyBio S column equilibrated with 40 mM Na-acetate buffer, pH 4.4 at 1 ml/min. Unbound proteins were eluted with 25 mL of the same buffer at 5 ml/min. Bound proteins were eluted by a 100 mL gradient of 0-0.5M NaCl in 40 mM Na-acetate buffer, pH 4.4, at 2.5 ml/min, followed by 25 mL of 1 M NaCl in 40 mM acetate buffer, pH 4.4. 2 mL fractions were collected during the elution. Selected fractions were pooled and desalted on a PD10-column equilibrated with 100 mM phosphate buffer pH 6.8. The desalted sample was referred to as *"Potato IEX S"*.

2.2.2.2 Anion Exchange Chromatography

17 mL of *"IEX S"* was applied onto a 5 mL BabyBio DEAE column equilibrated with 100 mM phosphate buffer, pH 7.4 at 1 ml/min. Unbound proteins were eluted with 25 mL of the same buffer at 5 ml/min. Bound proteins were eluted by a 100 mL gradient of 0-0.5M NaCl in 100 mM phosphate buffer pH 7.4 at 2.5 ml/min, followed by 25 mL of 1 M NaCl in 100 mM phosphate buffer, pH 7.4. 2 mL fractions were collected during elution. Selected fractions were pooled, desalted as mentioned above, and referred to as *"Potato IEX DEAE"*.

2.2.3 Analytical Methods

2.2.3.1 Protein Concentration

The protein concentration was determined for the four purified fractions *"Potato Crude"*, *"40% Potato Precipitate"*, *"Potato IEX S"* and *"Potato IEX DEAE"* by measuring the absorbance at 280 nm. The purified fractions were diluted in an appropriate buffer to calculate the approximate concentration of the sample. *"Potato Crude"* was diluted 320-fold in distillate water,"*40% Potato Precipitate*" was diluted 320- fold in 100 mM phosphate buffer pH 6.8. Similarly, the *"Potato IEX S"* was diluted 8-fold in the latter buffer mentioned. No dilution was required for the *"Potato IEX DEAE"* fraction. The protein concentrations for the purified fractions were calculated according to Eq 1.6. The molar extinction coefficient used was obtained from ProtParam with a value of 27 055 $M^{-1} cm^{-1}$, assuming that the protein of interest was either Serine Protease Inhibitor 1 (SPI-1) with a molecular weight of 24 kDa or Serine Protease Inhibitor 2 (SPI-2) with a molecular weight of 20.1 kDa (noted P58514 or P58515 in the UniProt Data Base). The pathway length for the calculations was 1 cm.

2.2.3.2 Activity Assay

The activity of trypsin was assessed by the hydrolysis of the chromogenic substrate BAPA. The molar extinction coefficient for the product para-nitroaniline, used for concentration calculations, was 8 800 M^{-1} cm^{-1} at 410 nm. The absorbance at 410 nm was measured for 100 seconds in presence of enzyme, substrate and inhibitor in 250 mM ammonium bicarbonate buffer, pH 7.8 in a total volume of 1 mL. The final concentrations for the assay were 0.25 μM trypsin, 0.005-0.5 mM BAPA and 5% (v/v) dimethyl sulfoxide (DMSO). The final concentration of the purified fractions, containing a protein with molar extinction coefficient 27 055 M^{-1} cm^{-1}, for the assay were calculated to 3.69 μM for *"Potato Crude"*, 1.54 μM for *"40% Potato Precipitate"* and 0.55 μM for *"Potato IEX S"*.

The activity for α-chymotrypsin was measured spectrophotometrically by hydrolysis of BTEE, with the differential molar extinction coefficient 964 $M^{-1}cm^{-1}$ at 256 nm for the product formation, hence used for the substrate concentration calculations. The absorbance at 256 nm was measured for 300 seconds in presence of enzyme, substrate and inhibitor in 50 mM Tris-HCl buffer, pH 7.8 in a total volume of 1 mL. The final concentrations for the assay were 0.125 μM α-chymotrypsin, 0.005-3 mM BTEE, 3% (v/v) methanol and 50 mM $CaCl_2$. The final concentration of the purified fractions, calculated as above, were 1.85 μM for *"Potato Crude"*, 0.38 μM for *"40% Potato Precipitate"* and 0.14 μM for *"Potato IEX S"*.

The activity of elastase was measured spectrophotometrically by the release of dyed fragments from the substrate complex E-CR. The molar extinction coefficient used for concentration calculations was 45 000 M^{-1} cm^{-1} at 495 nm. The absorbance at 495 nm was measured after 17.5 hours of end-over-end incubation at 37°C, followed by centrifugation at 5000 rpm for 15 minutes in room temperature. The assay was performed in 50 mM Tris-HCl buffer, pH 8.5. The samples contained aliquots of 0.5-15 mg substrate suspended in 6.0 mL of 50 mM Tris-HCl buffer pH 8.5. Together with a final concentration of 0.017 μM elastase and each purified fraction with final concentrations: 4.88 μM of *"Potato Crude"*, 5.08 μM *"40% Potato Precipitate"* and 0.18 μM of *"Potato IEX S"*.

The saturation curves for each enzyme were evaluated according to Michaelis-Menten Eq 1.2 with non-linear curve fit using MATLab, providing a corresponding V_{max} and K_m for each curve. The value for k_{cat} was calculated according to Eq 1.3. For the assay in presence of the inhibitor fractions, the saturation curves were fitted according to the same equation but providing an apparent value for K_m for estimating the inhibitor constant, K_i value, according to Eq 1.5.

2.2.3.3 Initial Binding Interactions

The interaction patterns between inhibitor, substrate and enzyme were investigated spectrophotometrically for trypsin and α-chymotrypsin with two different application orders. The first order was substrate mixed with inhibitor in appropriate buffer, followed by addition of enzyme. The second order was to mix inhibitor and enzyme with appropriate buffer followed by addition of substrate. The set up for the assay had the same wavelength, time, buffers and final concentrations as mentioned before for trypsin and α-chymotrypsin, but performed only with a final substrate concentration of 0.15 mM for both BAPA and BTEE.

2.2.3.4 Light Scattering

The light scattering analysis was performed for three samples; purified fraction referred to as *"Potato IEX S"* diluted to 0.011 mM in 100 mM phosphate buffer pH 6.8 as sample one. Sample number two contained 5 μM of trypsin, diluted in distilled water. The third sample contained a mixture of 100 μL of 0.022 mM *"Potato IEX S"* and 100 μL of 10 μM trypsin. 200 μL of each sample were applied to a 20 mL Superose 12™ column equilibrated with 250 mM ammonium bicarbonate, pH 7.8, at 0.5 ml/min. The light scattering, differential refractive index and UV-absorbance signals were monitored for 50 minutes and synchronized by the ASTRA software.

2.2.3.5 SDS-PAGE

The SDS-PAGE was performed using the PhastGel System. Sample preparations contained 10 μL 0.59 mM of *"Potato Crude"*, 0.31 mM of *"40% Potato Precipitate"* and 0.031 mM of *"Potato IEX S"*. Each purified fraction was treated with 10 μL of loading buffer including tracking dye, SDS and dithiotreitol (DTT). Samples were centrifuged at 2000 rpm for 1 minute in 4 ºC and heat treated in 95 ºC for 5 minutes, and applied according to manufacturers instruction on a homogenous 20% polyacrylamide gel, with SDS-PAGE buffer strips containing 0.2 M Tris-tricine buffer, pH 8.1 with 0.55% SDS. A polypeptide SDS-PAGE Standard was used as protein molecular weight marker, treated in the same manner as the samples. The gel was stained for 45 minutes with 0.2% Comassie Brilliant Blue R-250 in 40% methanol and 10% acetic acid, and destained for 60 minutes in 40% methanol together with 10% acetic acid in distilled water.

2.2.3.6 Mass Spectrometry

For mass spectrometry a selected SDS-PAGE band was analysed by in-gel digestion with trypsin according to standard operation procedure by LC-Orbitrap MS/MS at the MS Facility, SciLifeLab Uppsala University.

The Science for Life Laboratory Mass Spectrometry Based Proteomics Facility in Uppsala supported the mass spectrometry analysis of the purified fraction. Data storage was obtained and supported by Bioinformatics Infrastructure for Life Sciences.

2.2.3.7 Immobilisation

The immobilisation was performed in two steps; a coupling step with associated washing, and a blocking step with associated washing. For coupling, 1 ml of the purified fraction *"Potato IEX S"* was mixed with 1.0 g suction dried Bio-Works ACT media, and incubated end-over-end over-night at ambient temperature. After washing, the media was transferred to 1 M Tris-HCl buffer, pH 9.0 and incubated end-over-end over-night at ambient temperature for blocking of any remaining active groups. The medium was thoroughly washed and transferred to 20% ethanol.

The immobilisation was evaluated by end-over-end agitation of 0.2 g suction dried gel, in a final concentration of 0.125 μM trypsin and 2.5 mM BAPA in a total volume of 2 mL 250 mM ammonium carbonate buffer, pH 7.8 for 1 hour. The absorbance at 410 nm was measured for resin coupled with purified fraction *"Potato IEXS"* and for blank resin after centrifugation at 2000 rpm for 2 minutes in 4°C. The absorbance at 410 nm was also measured for the same concentrations and conditions but after 10 minutes of incubation.

3 Results

3.1 Protein Identification

3.1.1 Protein Purification

3.1.1.1 Protein Extraction

From 3.89 kg peeled, cut and mixed potato a fraction of *"Potato Crude"* was obtained with a total volume of 1246 mL.

From 1156 mL *"Potato Crude"* a fraction of desalted *"40% Potato Precipitate"* was obtained with a total volume of 45 mL.

3.1.1.2 Chromatographic Result

Figure 4 shows the gradient elution chromatogram of the cation exchange chromatography for 25 mL *"40% Potato Precipitate"* applied on to a 5 mL BabyBio S column. The first peak corresponds to the fraction *"Potato IEX S"* with a total desalted volume of 30.8 mL.

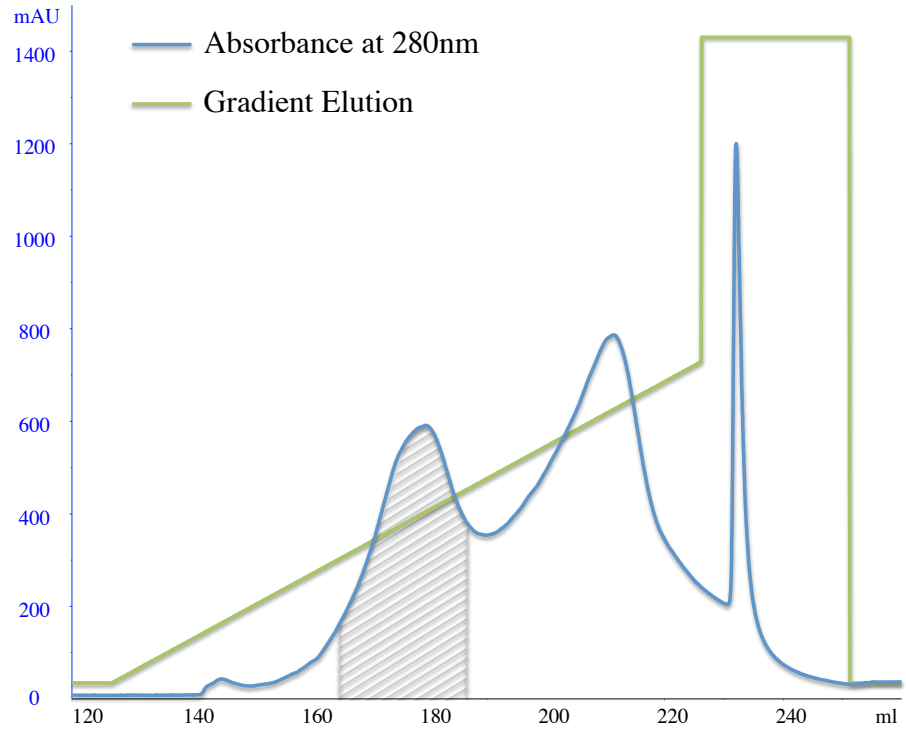

Figure 4: Chromatogram of *"40% Potato Precipitate"* gradient eluted form a Baby Bio S 5 mL column. 25 mL *"40% Potato Precipitate"* applied on a 5 mL BabyBio S column equilibrated with 40 mM Na-acetate buffer pH 4.4. Bound proteins were eluted by a 100 mL gradient of 0-5M NaCl in 40 mM Na-acetate buffer pH 4.4 followed by a 25 mL of 1 M NaCl in 40 mM Na-acetate buffer pH 4.4. 2 mL fractions were collected and pooled for the three peaks shown in the figure. The first eluted peak was pooled, desalted and referred to as *"Potato IEX S"*.

Figure 5 shows the gradient elution of 17 mL *"Potato IEX S"* applied on a 5 mL BabyBio DEAE. The small peak corresponds to fraction *"Potato IEX DEAE"* with a total desalted volume of 19.6 mL.

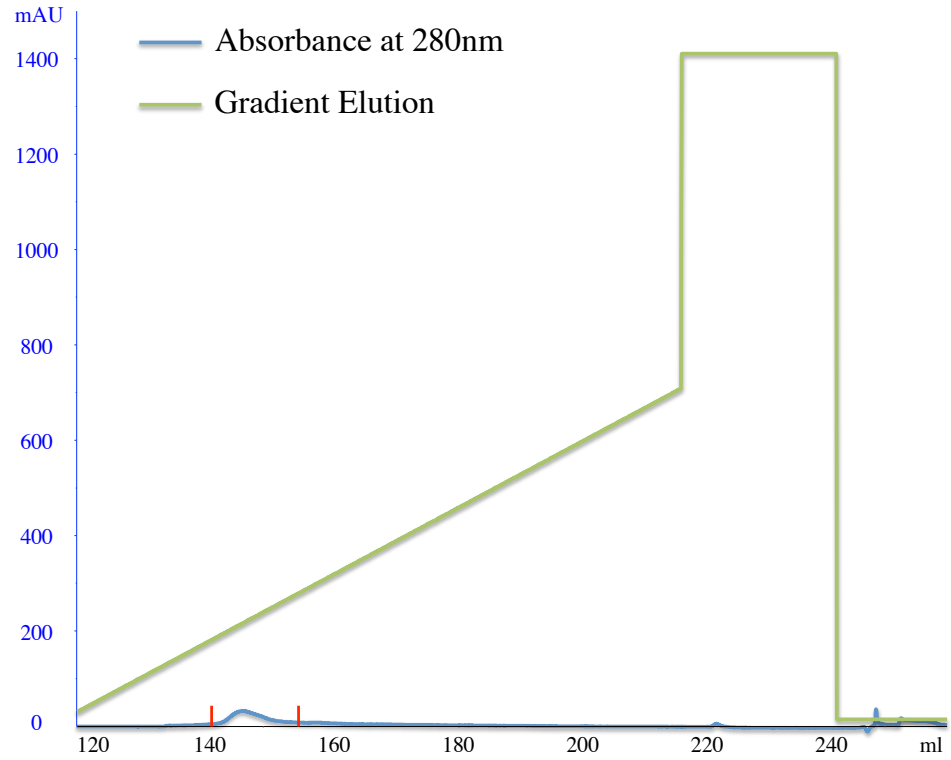

Figure 5: Chromatogram of *"Potato IEX S"* gradient eluted from a 5 mL BabyBio DEAE column. 17 mL of *"Potato IEX S"* applied on a 5 mL BabyBio DEAE column equilibrated with 100 mM phosphate buffer pH 7.4. Bound proteins were eluted in a 100 mL gradient of 0-5 M NaCl in 100 mM phosphate buffer pH 7.4, followed by 25 mL of 1 M NaCl in 100 mM phosphate buffer pH 7.4. 2 mL fractions were collected and pooled for one peak. The peak was pooled, desalted and referred to as *"Potato IEX DEAE"*.

3.1.1.3 Purification Scheme

The purification scheme is shown in figure 6, where the first step, protein extraction, gave the first fraction *"Potato Crude"* as described in section 2.2.1. The second step gave the *"40% Potato Precipitate"* after desalting, described in the same section. The third and last step, *"Potato IEX S"*, was obtained by cation exchange chromatography as described in section 2.2.2.

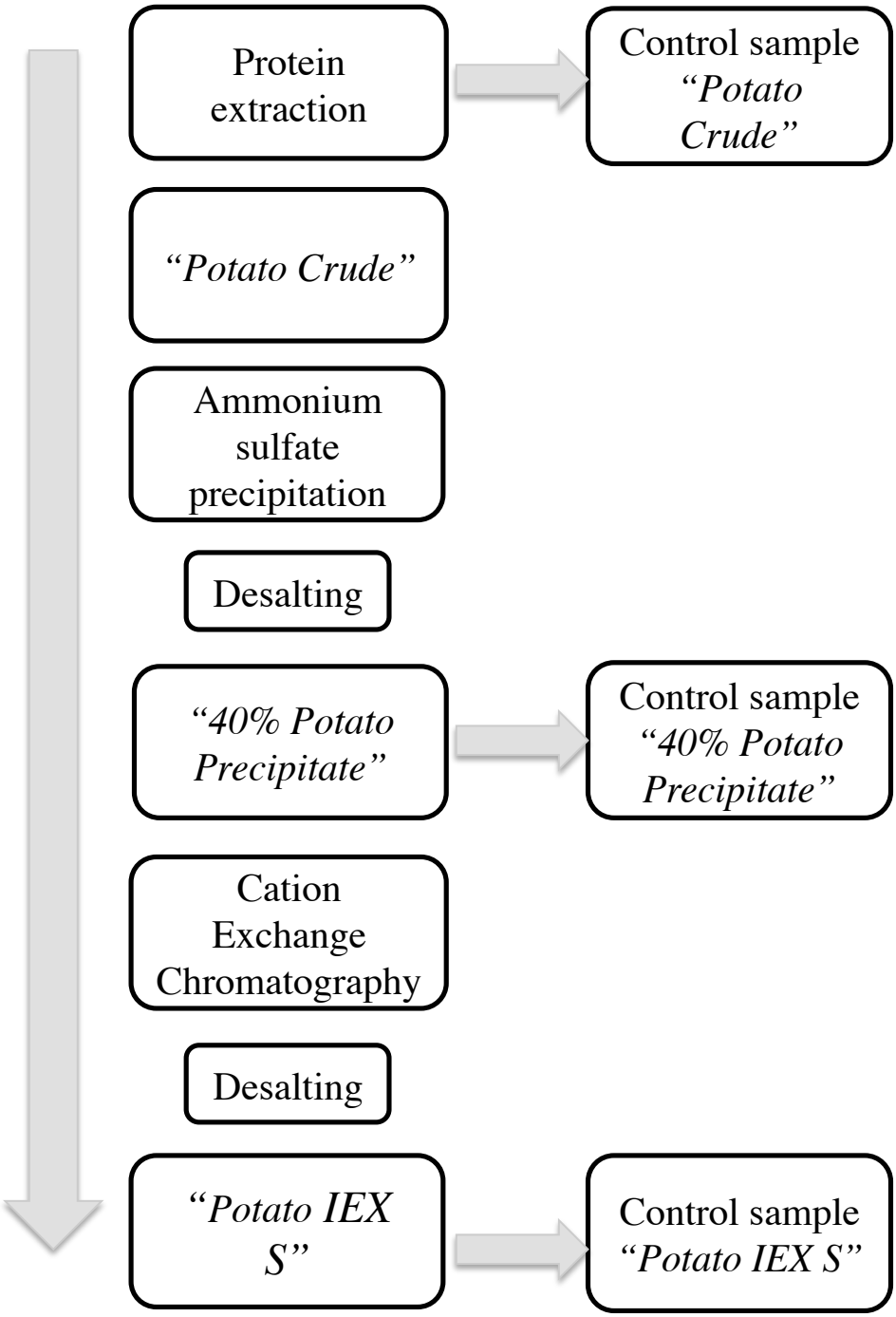

Figure 6: Overview of the purification scheme. *"Potato Crude"* was obtained from the filtrate from centrifuged and heat-treated peeled, cut and homogenized potato. *"40% Potato Precipitate"*, was obtained by 40% ammonium sulfate precipitation of fraction two. Last, *"Potato IEX S"* was obtained by cation exchange chromatography of fraction two eluted from a 5 mL BabyBio S column.

3.2 Protein Characterisation

3.2.1 Absorbance for Protein Concentration

The approximate molar concentrations and corresponding protein concentrations for each step are shown in table 1 and 2. The molar concentrations were calculated to 0.59 mM for *"Potato Crude"*, 0.62 mM for *"40% Potato Precipitate"*, 0.022 mM for *"Potato IEX S"* and 0.0019 mM for *"Potato IEX DEAE"*.

Table 1. Absorbance measurements at 280 nm, molar concentration and corresponding protein concentration for *"Potato Crude"* and *"40% Potato Precipitate"*. The absorbance measurements correspond to a stepwise dilution of each fraction to obtain values of a 1-0.01 linear range. The protein concentrations were calculated according to Eq 1.6 with a molar extinction coefficient of 27 055 M^{-1} cm^{-1}. The molecular weight of 24 kDa for SPI-1 and 20.1 kDa for SPI-2, respectively, was used.

Sample	Abs280 x20 dilution	Abs280 x80 dilution	Abs280 x160 dilution	Abs280 x320 dilution	Molar Conc. [mM]	Protein Conc. SPI-1 [mg/ml]	Protein Conc. SPI-2 [mg/ml]
"Potato Crude"	0.80	0.19	0.11	0.05	0.59	14.19	11.89
"40% Potato Precipitate"	0.9	0.22	0.11	0.05	0.62	14.76	12.36

Table 2. Absorbance measurements at 280 nm, molar concentration and corresponding protein concentrations for *"Potato IEX S"* and *"Potato IEX DEAE"*. The absorbance measurements correspond to a stepwise dilution of each fraction to obtain the linear range to calculate the protein concentration according to Eq 1.6 with a molar extinction coefficient of 27 055 M^{-1} cm^{-1}. The molecular weight of 24 kDa for SPI-1 and 20.1 kDa for SPI-2, respectively, was used. *No dilution required.

Sample	Abs280	Abs280 x2 dilution	Abs280 x4 dilution	Abs280 x8 dilution	Molar Concentration [mM]	Protein Conc. SPI-1 [mg/ml]	Protein Conc. SPI-2 [mg/ml]
"Potato IEX S"	0.34	0.19	0.11	0.074	0.022	0.53	0.44
"Potato IEX DEAE"	0.050	*	*	*	0.0018	0.044	0.037

3.2.2 Enzyme Activity Assay

3.2.2.1 Enzyme Kinetics Trypsin

Figure 7 shows the result of the initial rate to substrate concentration for trypsin and trypsin in presence of *"Potato Crude"*, *"40% Potato Precipitate"* and *"Potato IEX S"*. The values from the enzymatic assay were non-linear fitted according to Michaelis-Menten equation, Eq 1.2 evaluated in MATLab. Figure 7 also show the corresponding Line-weaver Burk plots for the results.

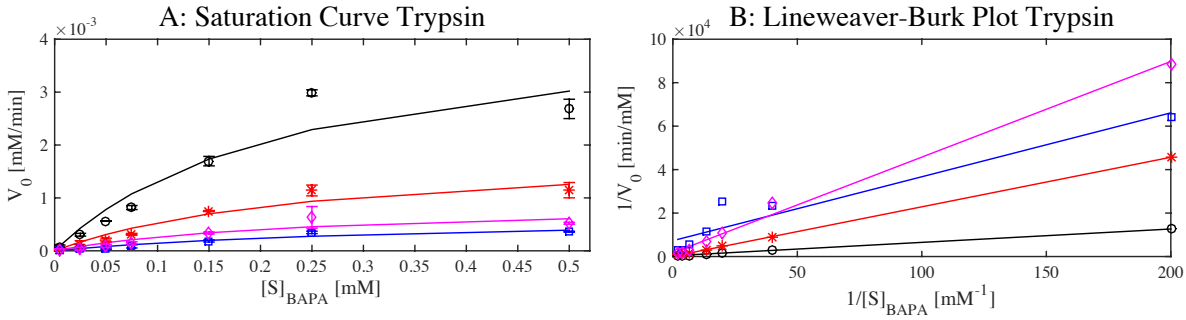

Figure 7: Saturation curve and Lineweaver-Burk Plot for trypsin of the initial rate at different substrate concentrations with corresponding inhibiting curves for *"Potato Crude", "40% Potato Precipitate"* and *"Potato IEX S".* (A) The four saturation curves correspond to trypsin (black) and trypsin in presence of three different samples: *"Potato Crude"* (red), *"40% Potato Precipitate"* (blue) and *"Potato IEX S"* (magenta). The curves were fitted according to Michaelis-Menten Eq 1.2 using MATLab; error bars for triplicate measurements are also shown in the figure. (B) Corresponding Lineweaver-Burk Plot for the saturation curves in figure 7A.

The estimated maximum velocity for the reaction, V_{max}, and the Michaelis constant K_m, for trypsin were 4.4 µM/min and 0.23 mM, respectively, with corresponding correlation coefficient R^2 of 0.91. The rate constant k_{cat} for trypsin was calculated, according to Eq 1.3, to 0.30 s^{-1}, resulting in a k_{cat}/K_m equal to 72.92 mM^{-1} s^{-1}. The values are shown in table 3.

Table 3. Kinetic values for trypsin. Calculated values for trypsin using MATLab and the Michelis Menten equation.

Enzyme	**V_{max} [µM min^{-1}]**	**K_m [mM]**	**k_{cat} [s^{-1}]**	**k_{cat}/K_m [mM^{-1} s^{-1}]**	**R^2**
Trypsin	4.4	0.23	0.30	1.27	0.91

In presence of the three samples *"Potato Crude"*, *"40% Potato Precipitate"* and *"Potato IEX S"* an apparent value for K_m was estimated according to the non-linear fit model also giving each fraction a value of K_i, the inhibitor constant. However, a ratio between k_{cat} and

the apparent K_m was also calculated. For all estimated saturation curves a corresponding correlation coefficient was determined. Values are shown in table 4.

Table 4. Overview table of $K_{m,app}$, k_{cat}/K_m, [I], K_i and R^2 for trypsin in presence of inhibitor fractions. The values were obtained from the non-linear fitted curve to Eq 1.2 in MATLab together with Eq 1.5. Data used were obtained from the enzymatic assay measurements for trypsin.

Step	$K_{m,app}$ [mM]	k_{cat}/K_m [mM^{-1} s^{-1}]	[I] [µM]	K_i [µM]	R^2
"Potato Crude"	0.259	1.14	3.7	33	0.94
"40% Potato Precipitate"	0.362	0.82	2.8	2.8	0.93
"Potato IEX S"	0.249	1.19	7.8	7.8	0.87

3.2.2.2 Enzyme Kinetics α-Chymotrypsin

Figure 8 shows the results of the initial rate over substrate concentration for α-chymotrypsin and corresponding curves in presence of each step containing inhibitor, as well as the calculated Lineweaver-Burk Plots.

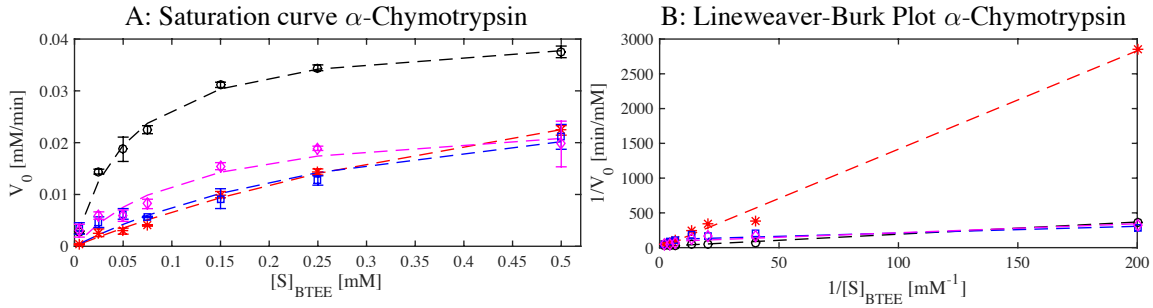

Figure 8: Saturation curve and Lineweaver Burk Plot for α-chymotrypsin together with inhibition curves for each step. (A) Saturation Curve for α-chymotrypsin (black), inhibition curve for *"Potato Crude"* (red), *"40% Potato Precipitate"* (blue) and *"Potato IEX S"* (magenta). (B) Lineweaver-Burk plot for α-chymotrypsin (black), inhibition curve for *"Potato Crude"* (red), *"40% Potato Precipitate"* (blue) and *"Potato IEX S"* (magenta).

The calculated V_{max} for α-chymotrypsin was 0.042 mM/min and corresponding K_m 0.058 mM, with a correlation coefficient of 0.99. According to Eq 1.3 an estimated k_{cat} value for the reaction was 42.56 s^{-1}, hence the ratio k_{cat}/K_m for α-chymotrypsin was 733.79 mM^{-1} s^{-1}. All calculated and estimated values for α-chymotrypsin are shown in table 5.

Table 5. Estimated kinetic values for α-chymotrypsin. Values for V_{max} and K_m were estimated from a non-linear fit curve to the Michaelis Menten equation using MATLab.

Enzyme	V_{max} [mM min^{-1}]	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [mM^{-1} s^{-1}]	R^2
α-chymotrypsin	0.042	0.058	42.56	733.79	0.99

For α-chymotrypsin, the apparent Michaelis Menten constant in presence of *"Potato Crude"*,*"40% Potato Precipitate"* and *"Potato IEX S"* were estimated. The apparent K_m value was used to calculate corresponding inhibitor constants, K_i, for each purified fraction. A ratio between k_{cat} and the apparent K_m was also calculated. All values for α-chymotrypsin are shown in table 6.

Table 6. Overview table of $K_{m,app}$, K_i and R^2 for α-chymotrypsin in presence of inhibitor fractions. The values were obtained from a non-linear fitted curve to Eq 1.2 in MATLab together with Eq 1.5. Data used are obtained from the enzymatic assay measurements for α-chymotrypsin.

Step	$K_{m,app}$ [mM]	k_{cat}/K_m [mM^{-1} s^{-1}]	K_i [μM]	[I] [μM]	R^2
"Potato Crude"	0.75	56.75	0.15	1.85	0.99
"40% Potato Precipitate"	0.36	118.22	0.074	0.38	0.90
"Potato IEX S"	0.12	354.67	0.13	0.14	0.94

3.2.2.3 Enzyme Kinetics Elastase

The result of the product formation over time for the hydrolysis of E-CR in presence of elastase and corresponding measurements for each step are shown in figure 9. The maximum velocity for elastase was estimated to 0.16 μM/hour and the corresponding Michaelis Menten constant to 9.7 μM.

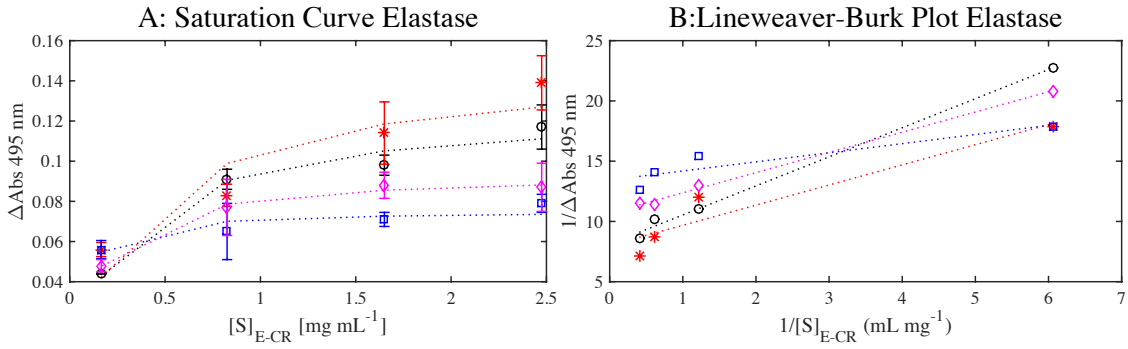

Figure 9: Saturation curve and Lineweaver-Burk plot for elastase. (A) Saturation curves for elastase (black) and corresponding fractions *"Potato Crude"* (red), *"40% Potato Precipitate"* (blue) and *"Potato IEX S"* (magenta). The curves correspond to the absorbance from substrate E-CR in presence of elastase over time for elastase saturation curve and in presence of substrate, enzyme and purified fractions containing the inhibitor. (B) Corresponding Lineweaver-Burk plots for the same curves.

The kinetic values for elastase in presence of the purified fractions are shown in table 7.

Table 7. Overview table of $K_{m,app}$, and R^2 for elastase in presence of each purified step. The values were obtained from the non-linear fitted curve to Eq 1.2 in MATLab. Data used for the curve fitting were obtained from the enzymatic assay measurements for elastase.

Step	**V_{max} [µM/hour]**	**$K_{m,app}$ [µM]**	**R^2**
"Potato Crude"	0.19	12	0.85
"40% Potato Precipitate"	0.096	1.9	0.79
"Potato IEX S"	0.12	4.8	0.99

3.2.2.4 Initial Binding Interactions

The results of the initial rate pattern for adding enzyme to a mixture of substrate and inhibitor are shown in figure 10 A and C. The result for adding substrate to a mixture of enzyme and inhibitor are shown in figure 10 B and D. Total concentration in the cuvette: trypsin: 0.15 mM BAPA, 0.25 µM trypsin and 0.15 mM BTEE, 0.125 µM α-chymotrypsin. The concentrations of the inhibitors for the different enzymes are the same as described in section 2.2.3.2.

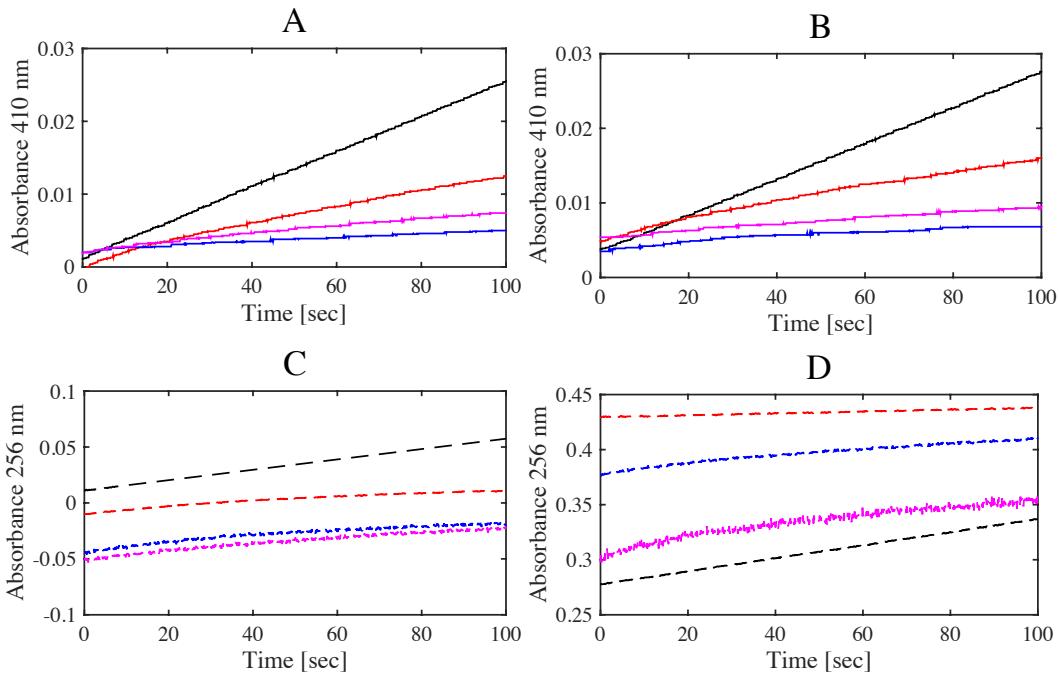

Figure 10: Initial rate patterns for trypsin and α-chymotrypsin by different mixing of enzyme, substrate and inhibitor. (A) Initial rate for mixing first substrate and inhibitor, trypsin without inhibitor (black),*"Potato Crude"* (red), *"40% Potato Precipitate*"(blue) and *"Potato IEX S"* (magenta), followed by adding trypsin. The final substrate concentration of BAPA was 0.15 mM and 0.25 µM trypsin (B) Same conditions for trypsin but enzyme and inhibitor was first mixed followed by adding substrate. (C) Equal assay as for (A) but 0.125 µM α-chymotrypsin as enzyme and 0.15 mM BTEE as substrate. (D) Equal assay as for (B) but α-chymotrypsin as enzyme and BTEE as substrate.

3.2.3 Light Scattering

Figure 11 shows detection of light scattering, differential refractive index and UV-light for trypsin and purified step *"Potato IEX S"* as well for the two as complex in a mixture. For trypsin and inhibitor alone one peak for the three parameters is detected at the very end of the analysis, in comparison to the complex where another peak also is visible earlier in the analysis, with a calculated molecular weight of 81 kDa. This result shows a strong indication of complex formation, but needs to be further confirmed.

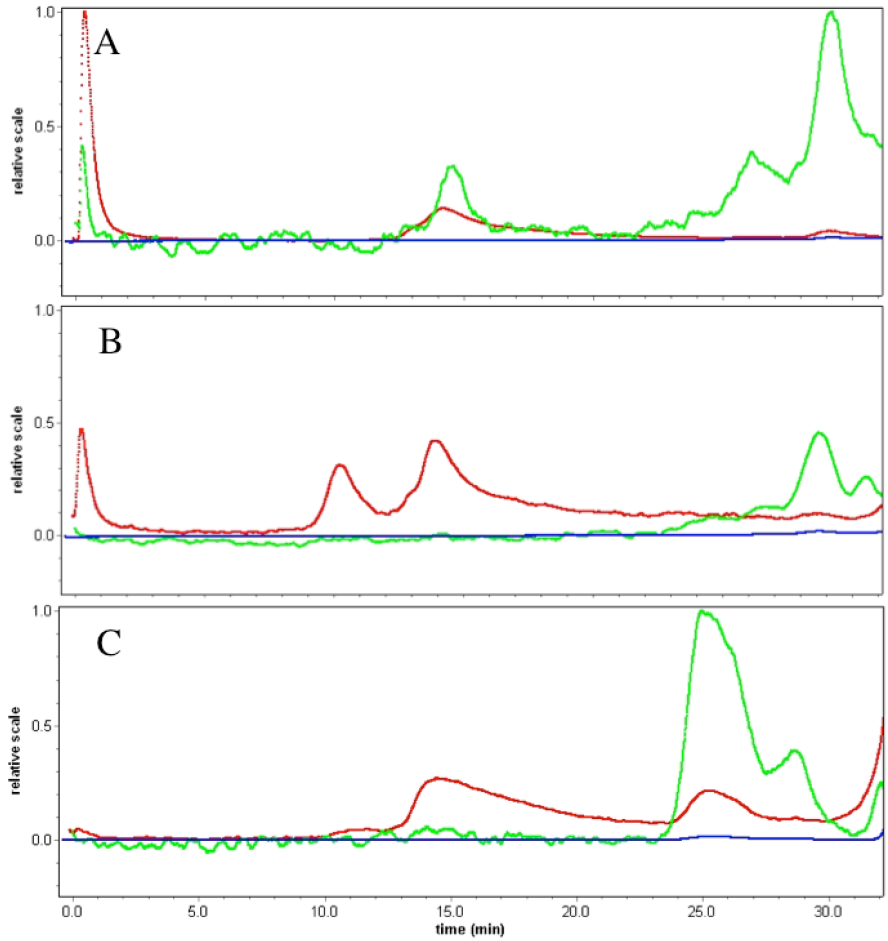

Figure 11: Detection for light scattering, differential refractive index and UV-signal of trypsin, purified *"Potato IEX S"* and the two together as a complex. (A) Signals of 200 µL of 5 µM Trypsin for detection of light scattering (red), differential refractive index (blue) and UV-signal (green). (B) Equal analysis for 200 µL of 0.022 mM *"Potato IEX S"*. (C) Analysis for corresponding concentrations in a total volume 200 µL of trypsin mixed with *"Potato IEX S"*.

3.2.4 SDS-PAGE

Protein bands from *"Potato Crude"*, *"40% Potato Precipitate"* and *"Potato IEX S"* evaluated on SDS-PAGE are shown in figure 12. Lane one contains a molecular weight standard of 26.6-1.4 kDa. Lane two contains 0.59 mM of the step referred to as *"Potato Crude"*, where one sharp band is visible above 26.6 kDa. Two bands are shown between 26.6 and 16.9 kDa, two bands between 16.9-14.4 kDa, one band at 3.4 kDa and two bands between 3.4-1.4 kDa. Lane three contains 0.031 mM of the step *"40% Potato Precipitate"* with two bands visible between 26.6-16.9 kDa, followed by one band between 16.9-14.4 kDa, one band at 3.4 kDa and one band between 3.4-1.4 kDa. Finally, lane four contains 0.031 mM of step *"Potato IEX S"* and shows two bands between 26.6-16.9 kDa, one band between 16.9-14.4 kDa and one band between 3.4-1.4 kDa.

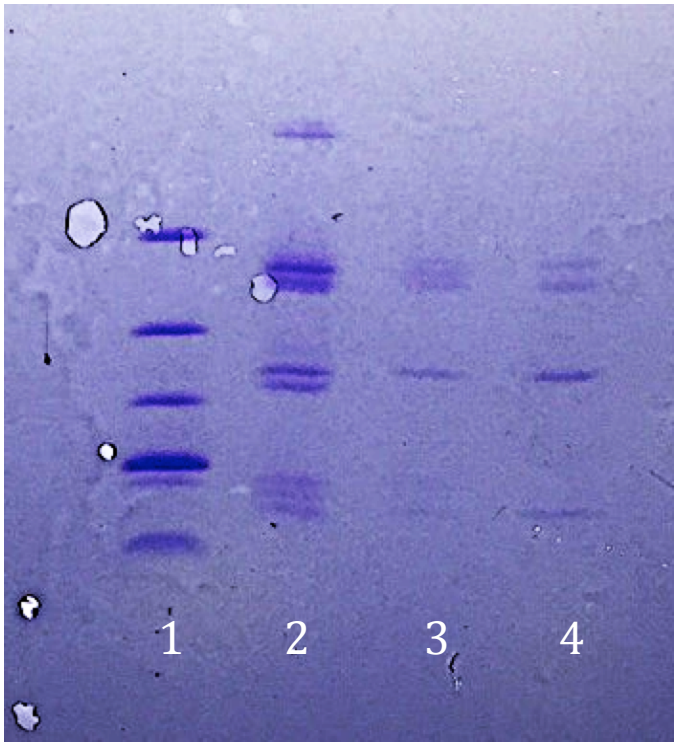

Figure 12: SDS-PAGE of *"Potato Crude"* (2), *"40% Potato Precipitate "* (3) and *"Potato IEX S"* (4) fractions, together with a molecular protein standard (1). Lane 1 shows a molecular weight standard with bands at corresponding molecular weights: 26.6, 16.9, 14.4, 6.5, 3.4 and 1.4 kDa. Lane 2 shows bands for 0.59 mM *"Potato Crude"*, lane 3 for 0.031 mM *"40% Potato Precipitate"* and lane 4 for 0.031 mM *"Potato IEX S"*.

3.2.5 Mass Spectrometry

The second band in lane 2 between 26.6 and 16.9 kDa was sent for mass spectrometry analysis using peptide mass fingerprinting with trypsin as digestive enzyme. The mass spectrometry results did show presence of two serine protease inhibitors: Serine Protease Inhibitor-1 and Serine Protease Inhibitor-2, values from the result shown in table 8. Figure 13 shows the FASTA sequence of the two proteins. The protein with the highest score and coverage, hence the most likely protein, was Serine Protease Inhibitor 2 with a molecular weight of 20.1 kDa.

Table 8. Overview of the result from the MS analysis. The table shows two potential proteins with their UniProt code, molecular weight, score and coverage from the MS analysis and a calculated pI value.

Protein	UniProt Code	Molecular weight [kDa]	Score	Coverage	Calculated pI
Serine Protease inhibitor-1	P58514	24.0	189.35	28.05	7.09
Serine Protease inhibitor-2	P58515	20.1	217.38	44.62	5.27

>sp|P58514|SPI1_SOLTU Serine protease inhibitor 1
OS=Solanum tuberosum PE=1 SV=2
MKCLFLVCLCLVPIVVFSSTFTSQNPINLPSDATPVLDVTGKELDSRLSYRIISTFWGAL
GGDVYLGKSPNSDAPCANGVFRYNSDVGPSGTPVRFIGSSSHFGQGIFENELLNIQFAIS
TSKLCVSYTIWKVGDYDASLGTMLLETGGTIGQADSSWFKIVKSSQLGYNLLYCPVTSTM
SCPFSSDDQFCLKVGVVHQNGKRRLALVKDNPLDISFKQVQ

>sp|P58515|SPI2_SOLTU Serine protease inhibitor 2
OS=Solanum tuberosum PE=1 SV=1
LPSDATPVLDVTGKELDSRLSYRIISTFWGALGGDVYLGKSPNSDAPCANGIFRYNSDVG
PSGTPVRFIGSSSHFGQGIFENELLNIQFAISTSKLCVSYTIWKVGDYDASLGTMLLETG
GTIGQADSSWFKIVKSSQLGYNLLYCPVTSSSDDQFCSKVGVVHQNGKRRLALVNENPLD
VLFQEV

Figure 13: FASTA sequence for Serine Protease Inhibitor 1 and 2 obtained from UniProt. The sequences had the UniProt Codes P58514 for Serine Protease Inhibitor 1 and P58515 for Serine Protease Inhibitor 2.

3.2.6 Purification Table

A purification table was obtained for the purified steps and their activity to trypsin; values are shown in table 9. An approximated specific activity was calculated according to the difference between the initial velocity with trypsin and the initial velocity in presence of inhibitor, normalized with the initial velocity of trypsin at a final substrate concentration of 0.25 mM BAPA. However, the total activity was calculated by the specific activity for the total protein mass for Serine Protease Inhibitor-2.

Table 9. Purification Table for *"Potato Crude", "40% Potato Precipitate"*, *"Potato IEX S"* and *"Potato IEX DEAE".* The purification table shows total volume, an approximated protein concentration, total protein mass, total activity, specific activity, yield and a purification factor for each fraction containing the inhibitor. * No measurements were performed for this step.

Step	Volume [mL]	Protein Concentration SPI-2 [mg/ml]	Protein mass SPI-2 [mg]	Total activity	Specific activity SPI-1 [Total Activity/mg inhibitor]	Yield [%]	Purification Factor
"Potato Crude"	1246	11.89	14811.07	2476452.05	167.20	100	1
"40% Potato Precipitate"	45	12.36	556.31	319590.92	574.48	12.91	3.44
"Potato IEX S"	30.8	0.44	13.55	19525.17	1440.98	0.79	8.62
"Potato IEX DEAE"	19.6	0.037	0.73	*	*	*	*

3.3 Technical Application

Table 10 shows the absorbance at 410 nm for one hour and ten minutes incubation for blank resin and resin coupled with *"Potato IEX S"*.

Table 10. Absorbance for immobilisation after 1 hour and 10 minutes. Each sample was end-over-end incubated with a final concentration of 0.125 μM trypsin and 2.5 mM BAPA in a total volume of 2 mL 250 mM ammonium carbonate buffer, pH 7.8. After the incubation time each sample was centrifuged for 2 minutes at 2000 rpm, in 4 °C.

Sample	Absorbance 410 nm, 1 hour	Absorbance 410 nm, 10 minutes
Blank resin	0.17	0.18
Resin coupled with *"Potato IEX S"*	0.18	0.15

4 Discussion and Conclusion

The aim of this master thesis was to develop efficient and easily scalable protocols for the purification and characterisation of protease inhibitors from *S. tuberosum*.

4.1 Protein Purification

To develop a scalable purification protocol one needs to consider both the availability of the protein, the over all yield of the process, together with time and cost for the over all procedure. However, the optimization of a purification protocol will be dependent on the protein of interest and the aim of the procedure.

Potato as a source of protein was both available and a natural source for the target protein for this master thesis project. The protein extraction procedure was found to be easily scalable, hence considered useful for large-scale applications. The stepwise ammonium sulfate precipitation was also considered easily scalable; hence fits into a scalable purification process in regards to both cost and yield. However, the target protein of this project did require being free of salt, thus a desalting step was added. The desalting step can be considered as a rate-limiting step when using larger scales, therefore consider revising if the fraction do not require being free of salt. However, the SDS-PAGE verified the presence of the protein of interest in the purified fraction, but declared not entirely purified, which did lead to a further purification step by using cation exchange chromatography.

When ion exchange chromatography is used one need to remember that different molecules can act in similar fashion under specific conditions: one single peak can contain more than only one molecule or protein. The cation exchange chromatogram obtained from present work did show three peaks eluted at different salt concentrations. The first peak was pooled as fraction *"Potato IEX S"*. However, the first two peaks were not entirely separated from each other. The conclusion was drawn that the pooled fraction was not a totally pure fraction of the protein of interest, which was later confirmed by SDS-PAGE. Still, the pooled fraction did contain the protein of interest, meaning that if one optimizes the cation exchange chromatography, one can possible obtain a pure pooled fraction of the protein of interest. Additionally, using ion exchange chromatography in larger scales is considered possible due to time, cost and yield. However, for this master thesis project a desalting step was added to obtain the second purified fraction, a rate-limiting step in larger scales but necessary for the purpose of this project.

An attempt for orthogonal ion exchange chromatography, with an additional purification step using anion exchange chromatography was tried out. However, the low protein concentration

and yield form the anion exchange chromatography and the time spent to perform the experiment made it difficult to include a second chromatography step in the purification procedure. Therefore, the fraction *"Potato IEX DEAE"* was excluded from both the purification process and the protein characterisations.

The protein purification process for this master thesis project was summarised to partly purify the protein of interest with a process that was considered scalable for all steps. The purification procedure was considered environmentally friendly due to the buffer of choice; the chemicals required were chosen to obtain an efficient economical procedure and the protein source was uncontroversial and natural. However, the process can be further optimized in regards to factors like purity and time and therefore also cost. As first suggestion to this, one needs to consider the priority of the purification process. If purity is the first priority, an additional step of for example size exclusion can be added. A more purified fraction may also be obtained by an optimization of the ion exchange chromatography by changing the binding conditions for the protein or the elution conditions. If one first prioritises the time, the desalting steps need to be reduced, which can be obtained by changing the elution conditions for the ion exchange chromatography to a pH elution. By not using the entire purification procedure one can also reduce the time of the purification procedure, but that will hence reduce the purity of the target protein.

4.2 Protein Characterisation

The protein characterization of this present work was obtained by determining different factors for *"Potato Crude"*, *"40% Potato Precipitate"* and *"Potato IEX S"*. First, the approximate protein concentration of the target protein was measured followed by kinetic studies for three different enzymes: trypsin, α-chymotrypsin and elastase. The interactions between inhibitor and enzyme were analysed with a light scattering analysis. A SDS-PAGE was performed followed by a MS analysis of the target protein. Last, a purification table was performed to evaluate the different purified fractions to trypsin.

4.2.1 Protein Concentration

The protein concentrations for the purified steps were approximately calculated according to Beer's Law based on the molar extinction coefficient for Serine Protease Inhibitor 1 and 2. However, the protein concentration was only an approximation because of the fact that the fractions were known to not be entirely pure. This can mislead the actual protein concentration due to impurities and other proteins with similar properties. Thus, this was the most accurate and user-friendly method for determining the protein concentration available for this project.

4.2.2 Enzyme Kinetics

The enzyme kinetics were evaluated under the assumption that the inhibitor of interest acts in competitive fashion, i.e. the maximal velocity remains constant while the Michaelis Menten constant, K_m, will be changed. Under these assumptions *"Potato Crude"* shows inhibition of trypsin and α-chymotrypsin. The *"40% Potato Precipitate"* and *"Potato IEX S"* show inhibition of trypsin, α-chymotrypsin and elastase. These conclusions are based on the calculated K_m values for enzymes alone compared to the calculated apparent K_m values in presence of the purified fractions. However, additional experiments can be performed to determine the type of inhibition. A more correct inhibition constant can be calculated when a high purity inhibitor with a known concentrations is prepared.

For trypsin the kinetic measurements for each step show an increase in the apparent K_m value compared to the original K_m measured for trypsin. This means that the purified fraction contained a molecule that binds to the active site of the enzyme, which leads to that a larger substrate concentration is required to reach the same maximum velocity under the specific conditions in presence of the purified fraction, i.e. the fractions contain a molecule that acts as a competitive inhibitor. The ratio k_{cat}/K_m was lower for each purified fraction in comparison to measurements without inhibitor, which verified that the enzyme efficiency was decreased in presence of the purified fractions that contained the inhibitor.

For the kinetic measurements of α-chymotrypsin the same pattern as for trypsin was shown. An increase in apparent K_m in presence of each step in comparison to the K_m calculated for the enzyme alone. The k_{cat}/K_m ratio was also decreased for all purified fractions, which verified that the fraction did inhibit the enzyme for all stages of the process. However, one needs to remember that these conclusions were under assumptions that the inhibitor did act as a competitive inhibitor.

The kinetics values for elastase were estimated values, approximated from the activity assay. Using E-CR as substrate for measuring the activity has difficulties in regards to distinguish between the substrate and the actual dye released. However, the saturation curves did verify that elastase did inhibit the steps *"40% Potato Precipitate"* and *"Potato IEX S"* in the purification process. The *"Potato Crude"* indicated to not inhibit elastase, which can possibly be a result of the high amount of other proteins in the fraction, i.e. the impurities. However, preliminary results indicated that the protein did inhibit elastase.

There were different initial binding interactions depending on the set up for the kinetic measurements for trypsin and α-chymotrypsin. By adding enzyme to a mixture of substrate and inhibitor, a slow equilibrium towards a linear production formation was obtained. The

inhibitor most likely did interrupt the enzymes while the substrate were trying to find a binding site on the enzyme, which can probably explain the first pattern. By adding substrate to a mixture of enzyme and inhibitor a faster equilibrium was obtained.
Most likely the complex of enzyme and inhibitor had already been formed before the substrate was added, hence the inhibitor-free enzymes were directly able to find a binding site on the substrate, hence faster equilibrium to a linear product formation.

4.2.3 Light Scattering

The light scattering experiment verified that the inhibitor forms a complex to the enzyme upon mixing. Accordingly, a molecule with a bigger molecular weight in comparison to the two molecules alone was formed and verified the complex formation. However, further experiments are required to evaluate the preferable stoichiometry of inhibitor bound to enzyme in the complex under specific conditions.

4.2.4 SDS-Page and MS analysis

The SDS-PAGE performed did verify the potential molecular weight of the proteins in the different purified fractions. It also verified that two proteins at an approximate molecular weight of 20 kDa were present in all purified steps. The purification procedure did purify the start sample from large size molecules and did purify the sample from some of the smaller molecules in the mixture. However, the gel revealed that the last purification step was not entirely pure, i.e. it did contain more than the target protein.

The MS analysis revealed the presence of two potential serine protease inhibitors in the second band between 26.6 and 16.9 kDa from the SDS-PAGE. The inhibitors were Serine Protease Inhibitor-1 and Serine Protease Inhibitor-2, with molecular weights of 24.0 and 20.1 kDa. However, the SDS-PAGE did show two bands with similar molecular weights, i.e. the band for analysis can have been contaminated with not only the second band but the first band as well. The serine protease inhibitor with the highest score and coverage for the MS analysis was Serine Protease Inhibitor-2 with a molecular weight of 20.1 kDa. Conclusions can therefore be drawn that the purified inhibitor most likely was that protein.

4.2.5 Purification Table

The purification table was based on the measurements for trypsin; the calculations were also based on the assumption that the purified protein was Serine Protease Inhibitor-2. The yield between *"Potato Crude"* and *"40% Potato Precipitate"* was 12.91%, which could be optimized by increasing the precipitation time, or optimizing the percentage of the salt added. The yield between *"Potato Crude"* and *"Potato IEX S"* was 0.79% meaning that the target

protein did have a low overall yield and hence the method can be optimized. The fold of purification was measured by the specific activity, and the *"40% Potato Precipitate"* was purified about 3 times in comparison to the start material and the *"Potato IEX S"* was purified about 9 times. The purification table shows that the purification process did work for this specific protein, however, optimizations can be performed to obtain a higher yield and purification factor.

4.3 Technical Application

The technical application of this project aimed to use the purified inhibitor as an immobilized carrier to a resin for inhibition to trypsin and other proteases. Preliminary experiments of the immobilization did indicate that there was a small degree of inhibition. However, further research need to be performed to validate the degree of inhibition and to optimize both the immobilization procedure and the method for evaluation.

4.4 Conclusion

The result from this master thesis shows that there was a serine protease inhibitor present in potato, which inhibits three essential enzymes in the human digestive system: trypsin, α-chymotrypsin and elastase. A scalable purification method showed that the inhibitor could be extracted from potato, concentrated by precipitation and partly purified by cation exchange chromatography. Purified fractions showed strong inhibition to trypsin and α-chymotrypsin and measurable inhibition to elastase. SDS-PAGE results are in accordance with the MS-analysis that suggested the protein of interest has a molecular weight of 20.1 kDa, and most likely is Serine Protease Inhibitor-2.

For further research and experiments the scalable purification process can be optimized by using an additional ion exchange chromatography step or by adding a size exclusion chromatography step. Finally, more experiments are required to optimize the immobilization of the inhibitor for technical applications.

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