OPTIMIZATION OF THE DOWNSTREAM PROCESS OF AFFINITY TAGGED PROTEASE CPCASP2

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Abstract

Removal of affinity tags after protein purification represents the bottleneck of affinity tag based processes. An authentic N-terminus is a strict requirement for biopharmaceuticals. Tag removal is usually performed using proteases cleaving off the tag at a specific sequence engineered in between the tag and the protein of interest. Many proteases cleave only after a specific amino acid making it very hard to leave an authentic N-terminus, since the protein of interest often does not have these specifically needed amino acids naturally at the N-terminus. Caspase 2 was identified to cleave the protein sequence after any amino acid and thus can produce an authentic N-terminus in any protein. This property represents a huge advantage when setting up a platform purification process for affinity tagged proteins especially in biopharmaceutical industry.

In this work, the purification process of circular permutated caspase-2, tagged with a poly-histidine tag, was characterized and optimized. For process characterization a FRET-based analytical assay was adapted and optimized to quantify enzymatic activity in crude samples. Additionally, it was evaluated whether the buffer exchange with subsequent chromatographic polishing could be replaced by a mixed mode chromatography step. The process optimization approach could reach an overall process yield, which was more than doubled from 17% to 38%.

Kurzfassung

Abtrennung des Affinitäts-Tags repräsentiert einen der limitierenden Faktoren in affinitätsbasierten Prozessen. Bei Bio-Pharmazeutika ist ein athenischer N-terminus strikt erforderlich. Die Entfernung des Affinitäts-Tags erfolgt mithilfe einer Protease, die die Proteinsequenz spezifisch zwischen Affinitäts-tag und Zielprotein schneidet. Viele Proteasen schneiden die Sequenz nur nach einer bestimmten Aminosäure. Daher ist es oft schwer einen authentischen N-Terminus zu erzeugen, da die meisten Proteine die speziell benötigte Aminosäure nicht natürlich am N-Terminus tragen. Caspase 2 kann die Proteinsequenz nach jeder beliebigen Aminosäure schneiden und damit bei jedem Protein einen authentischen N-Terminus erzeugen. Diese Eigenschaft repräsentiert besonders in der Biopharmazeutischen Industrie einen immensen Vorteil, wenn ein affinitätsbasierter Plattform-Prozess für entwickelt werden soll

In dieser Arbeit wurde der Aufreinigungsprozess von zirkulär permutierter, Histidin-getaggter Caspase 2 charakterisiert und optimiert. Um den Prozess ausreichend zu charakterisieren wurde ein FRET basiertes analytisches Verfahren weiterentwickelt und optimiert, um besonders stark verunreinigte Proben quantifizieren zu können. Zusätzlich wurde untersucht, ob die zweite Chromatographie mit vorhergehendem Puffer-Tausch ersetzt werden können durch eine multimodle Chromatographie. Die Optimierung des Prozesses könnte die Prozessausbeute von 17% auf 38% mehr als verdoppeln.

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1 Introduction

1.1 *Escherichia coli* as expression host

Since the beginning of recombinant protein technology, the gram negative enterobacterium *E. coli* played a major role as expression host. The first ever marketed recombinant protein, recombinant human insulin, was produced in *E. coli* [1]. The advantages to use *E. coli* as an expression system include the ability of rapid growth, inexpensive media, well characterized genetics and physiology [2], how cell density culture and the possibility of a fast and easy plasmid transformation [3]. However, especially when producing complex protein, *E. coli* has some limitations. As a prokaryote, it is not able to perform post-translational modifications like glycosylation, which are of major importance for various biological functions [1]. Disulphide bonds can only be formed, when the protein is transferred to the oxidizing environment of the periplasmic space [4]. The majority of recombinant proteins produced in *E. coli*, are nevertheless expressed in the cytoplasm. This is due to a higher possible protein yield as well as an easier plasmid construct [5]. Cytoplasmic expression often leads to formation of inclusion bodies due to an inappropriate chaperone system as well as limited solubility [6]. Inclusion bodies are inactive aggregates of recombinant proteins [5]. Formation of such can be advantages since they can be isolated rather easily via centrifugation and usually contain the recombinant protein in a high purity. The major drawback however, is the need of refolding since inclusion bodies are not biologically active [4, 5]. This step is often costly and provides usually very low yields and therefore makes production of recombinant proteins in form of inclusion bodies a rather unattractive strategy [5]. An approach to avoid the formation of inclusion bodies is to increase the solubility of the protein. This can be achieved by fusion of the target protein to a solubility tag. Among enhancement of solubility, fusion tags can also be advantageous in protein purification.

1.2 Fusion tags

Fusion tags are peptides that are linked either N- or C-terminal to the protein of interest (POI) [7, 8]. They can vary greatly in length ranging from less than 1 kDa for poly-arginine and poly-histidine tags to approximately 55 kDa for N-utilization substance A (NusA) [8]. Basically, two groups can be differentiated, namely solubility tags and affinity tags for purification [9]. The division into those groups however is not very strict since many solubility enhancing tags have an affinity towards a specific substrate. Additionally, there are affinity tags that also influence the protein solubility. In Figure 1 the basic structure of a tagged protein is shown. The main components are the tag itself, a linker sequence and the protein of interest.

Figure 1: Schematic illustration of an N-terminal tagged fusion protein. Between tag and POI a linker sequence is inserted. To separate tag and POI by enzymatic cleavage, the enzyme is specifically cleaving at the linker sequence.

Often a solubility enhancing tag is combined with an affinity tag to yield a soluble protein which is easy to purify [8, 10].

The main function of affinity tags is to aid a common process for protein purification [11], since separation of the protein of interest depends nearly exclusively on the affinity of the tag to a substrate and no longer of the individual characteristics of the protein. Affinity tags bind to the substrates in a highly specific manner and hence leading to efficient purification processes [9]. Especially the fact that the purification does hardly depend on the protein itself but rather on the tag makes affinity-based protein purification an excellent candidate for setting up a platform purification process.

1.2.1 Poly histidine tag (His-tag)

The most frequently used affinity tag however is the poly histidine-tag [9, 11]. It consists of a sequence of multiple histidine residues ranging from 2 up to 10, with 6 being the most commonly used variant [12]. Purification of recombinant proteins carrying a His-tag is performed using immobilized metal affinity chromatography (IMAC). Histidine shows a strong affinity towards transition metal ions as Zn^{2+}, Cu^{2+}, Co^{2+} or Ni^{2+} [10, 11, 13]. Binding occurs because the imidazole ring of histidine acts as electron donor and thus forms a coordinate bond with the transition metal. The metal ions are immobilized onto a stationary phase via a linker. The linker can for example be nitrilotriacetic acid (NTA) or iminodiacetic acid (IDA) [12]. The most popular combination of linker and metal is Ni^{2+}-NTA [8, 10].

Elution of the bound fusion protein is affected by using imidazole in concentrations up to 500 mM. The imidazole in the elution buffer competes with the imidazole group of the histidine residues of the tag for the binding sites and thus leads to dissociation of the tagged protein [14].

One disadvantage of purification of his-tagged protein is that other compounds may also bind to the immobilized metals and co-elute with the target protein. The contaminants also binding to the stationary phase are proteins with naturally occurring histidine or cysteine rich regions [8, 11, 13]. A possibility to try to minimize this effect is addition of low concentrations imidazole to the equilibration and wash buffer during purification [10].

Although effects of His-tag on the protein of interest are usually very low, removal of the tag may be needed. For therapeutic proteins, an authentic N-terminus must be given and thus complete tag removal must be performed [12].

1.2.2 Tag removal

Removal of fusion tags may be necessary since tags can interfere with the biological function of the protein or be immunogenic. It can either be performed using chemical or enzymatic methods [8].

Chemical methods are most often less expensive and reagents are easier to separate from the cleavage products [8]. The most commonly used reagent is cyanogen bromide (CNBr) [8, 10, 11]. A methionine residue between the tag and the protein of interest is needed, where the reagent than non-specifically performs breakage of the peptide bond at the C-terminus. The protein of interest must not carry any internal methionine residues since this would lead to internal cleavage of the target protein However, tag removal using CNBr requires harsh conditions as high concentrations of guanidinium chloride and trifluoroacetic acid and is thus not suitable for a broad variety of proteins. Another drawback is the limited sequence specificity [8].

Therefore the usual method to separate protein and fusion tag is by the use of an exo- or endoprotease with the latter being the more common one [15]. Enzymatic methods are preferred since they work under milder conditions and are more specific. The ratio of enzyme to protein is often very high resulting in high process costs, which represents, next to the long incubation times needed, the major disadvantages of these methods [11].

Fusion tag cleavage by proteolysis requires a specific cleavage site. This sequence must be additionally inserted between fusion tag and target protein [8, 9]. The most frequently used enzymes include enterokinase, tobacco etch virus (TEV) protease, thrombin, and factor Xa. All of these belong to the class of endoproteases [9-11, 13]. If the protein to be produced requires a native N-terminus, enterokinase may be one of the enzymes of choice since it does not need a specific amino acid residue at the C-terminal side of the cleavage site. Hence, it is possible to create a totally tag free protein [11, 15]. Having the tag cleaved off completely and thereby creating an authentic N-terminus is of special interest for biopharmaceuticals since in pharmaceutical industry an authentic N-terminus is a strict requirement. Therefore, many proteases are not suitable to be used in purification processes of biopharmaceuticals, which creates a high request for proteases being able to cleave the sequence after any amino acid leaving the protein with an authentic N-terminus.

After successful cleavage, the cleavage products as well as the cleaving enzyme need to be separated from the protein of interest. A common approach is the additional tagging of the enzyme with the same affinity tag as the protein of interest.

Consequently it is possible that after cleavage, the protein solution is purified using the same chromatographic method as with the initial purification of the protein of interest. In this case however, the tagged cleavage enzymes as well as the removed tag are retained by the stationary phase but not the protein of interest since it is no longer fused to the tag [11, 15]. This yields a high purity separation of protein of interest, leaving the column unretained, from the removed tag and the tagged enzyme, which bind the column due to affinity interactions.

1.3 cpCasp2

In this work, purification process of the caspase-2 variant cpCasp2 was optimized. The basic idea is the use of cpCasp2 to separate fusion tag and proteins. The enzyme itself as well as the target proteins is tagged with a 6xHis-tag. This allows purifying the enzyme and additionally removing of the cleavage products using IMAC for both steps.

1.3.1 Wild type caspase-2

Caspases are cysteine aspartic proteases that cleave their substrate following an aspartate residue [16-18]. In nearly all healthy cells caspases are present in an inactive precursor from. They are key regulators of apoptosis (caspase-2, -3, -7, -8, -9, -10) and other cell death events as well as in inflammation (caspase-1, -4, -5, -11, -12) [18]. Activation of pro-caspases is thought to be a process of self-cleavage after dimerization involving a so called long pro-domain [17].

Caspase-2 is activated by various apoptotic. To be fully active, caspase-2 dimerizes and performs auto-cleavage [16]. Although it was one of the first caspases to be discovered, its exact functions still remain unclear [17]. However, it is thought that it is involved in not only apoptosis but also in tumour suppression, reduction of oxidative stress, development and differentiation as well as in aging processes [16, 18].

Caspase 2 is especially interesting in protein purification processes since it is able to cleave a protein sequence regardless of the amino acid C-terminal of the cleavage site. For therapeutic proteins fused to an affinity tag caspase 2 represents an

optimal cleavage enzyme for tag removal since these biopharmaceuticals require an authentic N-terminus.

1.3.2 cpCasp2 structure

cpCasp2 is a circularly permutated (cp) caspase-2 variant. Circular permutation means the linkage of N- and C-terminus with an appropriate linker and cleavage of the protein at another site. Thus, two new termini are created. This alters the amino acid order but not the tertiary structure of the protein. [19].

Although different other caspases were already altered by circular permutation [20, 21], caspase-2 was first addressed by Petra Engele [19]. Figure 2: Comparison of wild type caspase-2 and circularly permutated caspase-2. Figure from Engele [19] shows a comparison of the wild type procaspase-2 and the circularly permutated variant cpCasp2. The N-terminal CARD domain was removed, position of small and large subunit was switched and a 6xHis-tag was introduced to allow easier purification. Exchange of small and large subunit was performed to create a constitutively active enzyme [19]. The constitutive activity is especially important to assure that the cpCasp2 performs substrate cleavage without the need of enzyme activation by cleavage and structural re-arrangement.

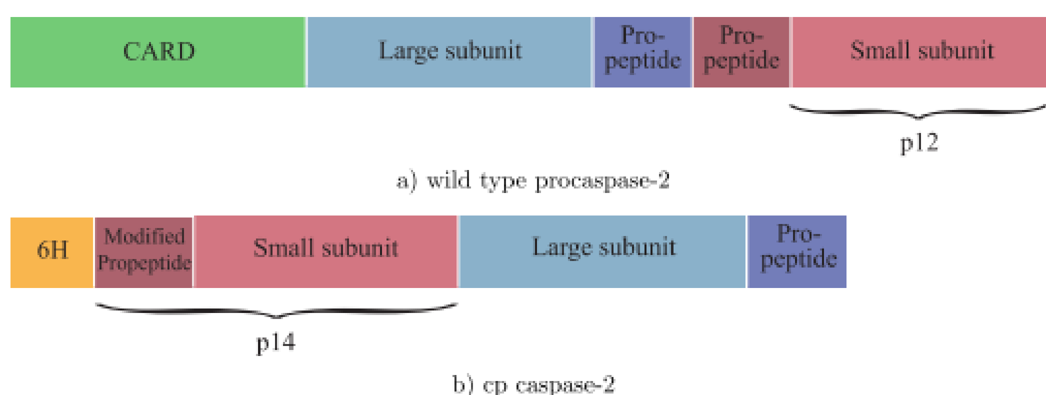

Figure 2: Comparison of wild type caspase-2 and circularly permutated caspase-2. Figure from Engele [19]

1.3.3 cpCasp2 downstream process

In Figure 3 an overview of the cpCasp2 downstream process is shown. Figure 3A shows the current process. The first unit operation is high pressure homogenization (HPH) to lyse the *E. coli* cells to release the intracellularly expressed enzyme followed by separation of cell debris by centrifugation and filtration. As a first purification step, a capture chromatography in IMAC mode is performed. Afterwards buffer is exchanged by diafiltration. This is necessary to achieve the conditions required for the next step, which is cation exchange chromatography (CEX). The elution buffer of the CEX also functions as formulation buffer. As an alternative approach to replace the buffer exchange and CEX, a mixed mode chromatography step is integrated. The process is shown in Figure 3B.

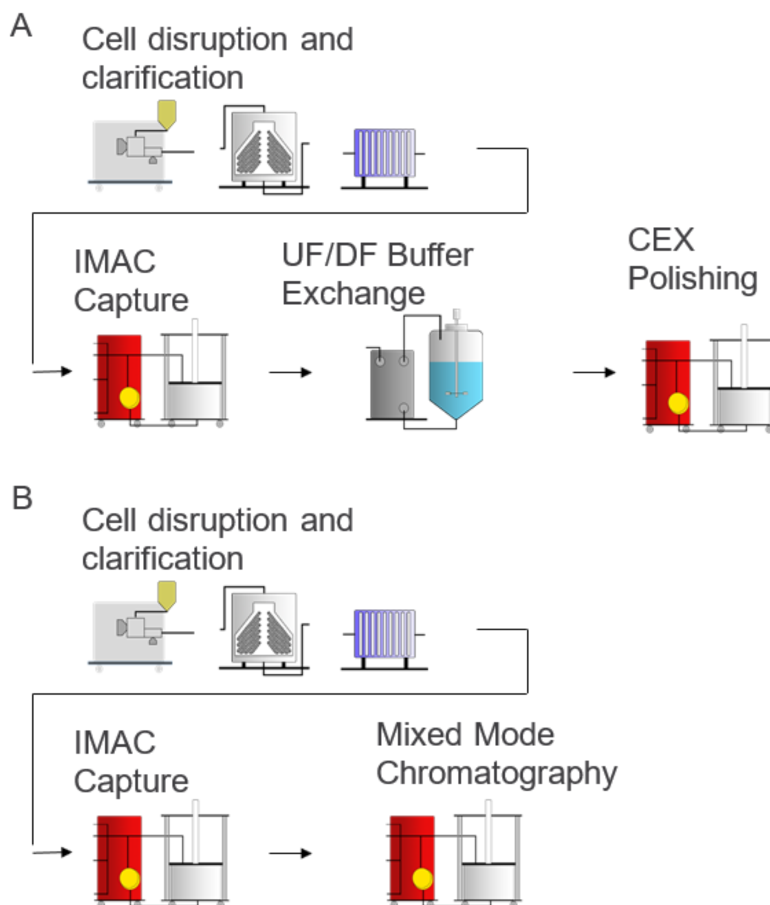

Figure 3: Process scheme of the downstream process of cpCasp2 (A). UF/DF Buffer exchange and CEX polishing is tried to be substituted by a mixed mode chromatography step (B)

1.3.3.1 High pressure homogenization

The protein of interest, cpCasp2, is expressed in the cytoplasm as mainly soluble protein. Although to some extent inclusion bodies containing the enzymes are present, they were not taken into further account since the necessary refolding process is usually of low yield. To release the soluble cpCasp2 from the host organism, it is necessary to disintegrate the *E. coli* cell. In this process disruption is performed using high pressure homogenization.

Cell disruption methods can be grouped in non-mechanical like chemical, physical, enzymatic or mechanical methods. In large scale production, mechanical methods like HPH or bead milling are most common due to their shorter process times, possibility to be operated continuously and generic application across cell types [22, 23].

In HPH, a pump pressurizes the cell suspension and forces it through a valve where the pressure then is suddenly released. Additionally the suspension impacts a solid surface, the impact ring [22]. Disruption of cells is achieved by a combination of mechanisms. The sudden pressure-drop and the impingement of the cells on the impact ring of the homogenizer are thought to be the main causes of cell breakage. Also, other factors such as viscous shear stress and cavitation play a role. Kinetics of cell disruption follows a first order kinetic with respect to the operating pressure and number of passages through the valve [22, 23]. Temperature and density of the cell suspension have none or very little influence on the cell breakage [22].

Although HPH is can be fast and easily operated and is applicable to nearly any kind of cell [22, 23], there are certain limitations. Efficiency of disruption is directly dependent on the fermentation conditions, which makes it hard to independently address the performance. The goal during cell disruption is the breakage of the cell envelope consisting of a cell wall and one or more membranes. Composition of the cell wall changes with growth phase and is the strongest during the stationary phase. This is mainly because there the peptidoglycans are more highly crosslinked. This suggests that during the stationary phase, the cells are more rigid and an even higher energy is needed to break them [23]. An effect of the high energy input is the amount of heat generated which increases the temperature of the cell suspension

during homogenization. This may lead to protein degradation and therefore sufficient cooling must be provided. Nevertheless, especially in large scale production, HPH is still the method of choice for cell disruption [22, 23].

1.3.3.2 IMAC capture

As already described in chapter 1.2.1 Poly histidine tag (His-tag), the basic mechanism of IMAC is the affinity of the certain amino acids like histidine towards transition metal ions. The metal ion is immobilized to a matrix via a linker. The most commonly used ones for affinity purification of proteins are IDA and NTA. Whereas IDA is trivalent, NTA is tetravalent due to an additional carboxymethyl group. Advantages of IDA over NTA are a higher metal loading density since it is a smaller molecule, lower costs and a lower amount of imidazole needed to elute the target protein. Lower imidazole requirements result from the less strong binding and lead to a decrease in overall process cost. A higher loading density of metal ions can generate higher yields, since more of the target protein can bind. However as already mentioned the purity then is lower [24]. A lower purity in IDA-based processes may also result from the fact that with 3 free binding sites, IDA is more likely to bind impurities than NTA with only 2 free binding sites.

IMAC often is used as one step purification method for his-tagged proteins and therefore NTA may be the preferred as a linker due to its higher purity making a subsequent polishing step often redundant.

The metal ions immobilized for purification of his-tagged proteins are usually Co^{2+}, Cu^{2+}, Zn^{2+} or Ni^{2+}. Affinity and specificity are inversely proportional with Cu^{2+} having the highest affinity but the lowest specificity and oppositely Co^{2+} with the lowest affinity but highest specificity. Ni^{2+} and Zn^{2+} are in between providing a kind of compromise between these two properties [24]. In his-tagged proteins produced in *E. coli*, Zn^{2+} may have an advantage since the host cell proteins do have a lower affinity towards Zn^{2+} [25].

Although IMAC is compatible with a broad spectrum of chemicals and can be operated in both oxidizing and reducing conditions, no chelating agents must be used. Chelating agents like Ethylenediaminetetraacetic acid (EDTA) form complexes with

the metal ions making them unable to bind the protein of interest and thus interfere with the purification mechanism. Other potential chelating groups like ammonium salts, Tris or certain amino acids can also be used within a certain concentration range. The reducing agent dithiothreitol (DTT) can be advantageous in purification of proteins since it partially reduces the protein making it possibly more accessible for binding. However concentrations should not exceed 10 mM [14].

Protein purification using IMAC leads to a high purity due to the high affinity and specificity of the method. Still, there are some contaminating host cell proteins that co-elute with the protein of interest. In *E. coli*, this can be proteins with histidine clusters or other metal binding motifs or proteins binding either the agarose matrix or another protein already adsorbed to the stationary phase. Avoiding these contaminating proteins can be achieved by engineered strains lacking the contaminating proteins, changing the matrix to a non-agarose material or adjusting the ratio of his-tagged protein to stationary phase, to name some examples. Addition of low concentrations of imidazole to the binding buffer is done to avoid binding of host cell proteins with increased histidine or cysteine residues on their surface [14]. Those proteins often do not bind very strongly and hence are replaced by the imidazole. Purification of his-tagged proteins often involves tag cleavage followed by IMAC, but in flow-through mode. This means that the removed tag binds the stationary phase and the tag-free protein, without any affinity to the stationary phase, leaves the column unretained directly after loading. This step also increases the purity further since contaminating proteins again bind the stationary phase, whereas the POI, now lacking the his-tag, is in the flow-though.

IMAC provides an indispensable method for the purification of his-tagged protein. Since the binding occurs between the histidine residues of the tag and the immobilized metal, the biochemical characteristics of the protein of interest usually do not play a major role in binding and must therefore not be known exactly. This can make IMAC a unit operation for a platform process for the purification of his-tagged proteins. Often only one purification step using IMAC leads to sufficient purity due to the high affinity and specificity of this method. Besides that a high affinity and specificity, low costs, simple operation and linear scalability make IMAC suitable for small scale as well as industrial scale purification of his-tagged protein [14].

However, especially in biopharmaceutical industry, affinity purification processes are not very common yet. The main problem is the removal of the affinity tag after initial purification. For biopharmaceuticals, an authentic N-terminus is a strict requirement. Since hardly any proteases that able to create such an authentic N-terminus are established in industrial scale, these processes are yet to be transferred from laboratory scale to large scale.

1.3.3.3 Mixed mode chromatography

As shown in Figure 3, one possible optimization approach is the substitution of buffer exchange and subsequent polishing chromatography by a mixed mode chromatography. Prerequisite for the implementation of this unit operation directly after capture by IMAC is a compatibility of the IMAC elution buffer as mixed mode binding buffer. Otherwise buffer must be exchanged and there is little benefit of replacing the diafiltration and polishing chromatography by a mixed mode chromatography.

Mixed mode chromatography, in comparison with traditional, single mode chromatography uses two or more adsorption mechanisms instead of one. However also in traditional chromatography so called secondary interactions were investigated. Hereby they are usually are tried to be avoided. In ion exchange chromatography, for example there is sometimes also some hydrophobic interaction present. Mixed mode chromatography actively uses this secondary interaction to increase selectivity of the stationary phase. Both interactions should contribute to the interaction of the solutes with the stationary phase and should ideally be not too weak [26, 27].

Mixed mode chromatography can be a powerful tool to purify proteins with high selectivity and high binding capacity. With combining two single mode chromatographic steps into one, it is also possible to reduce the consumption of material and waste production [26].

In practical terms however, mixed mode chromatography faces several challenges. A deep understanding of the underlying mechanisms in mixed mode chromatography is helpful for the development of such a method since mechanisms in mixed mode chromatography are complex. Therefore setting up a mixed mode

chromatography process requires intensive development and optimization [27]. This may probably the most prominent drawback of this method.

1.4 FRET-assay

To determine whether the optimisation approaches were successful, a method for quantification of cpCasp2 is needed. For pure samples RP-HPLC is the method of choice. Crude samples, such as the lysate after high pressure homogenisation as well as the flow-through fractions of the IMAC Capture step, cannot be quantified using this analytical method. Therefore, an assay similar to the one described by Zauner et al. [28], but using another mechanism of FRET, was developed. The assay uses the principle of fluorescence resonance energy transfer (FRET) for detection of protease activity. It is highly a specific method since it uses enzymatic cleavage as well as very sensitive due to fluorescence detection. Sensitivity of FRET based assay was demonstrated by Zauner et al [28].

Figure 4 shows the mechanism of the assay used in this work. If a fluorophore is in very close contact with a quencher, like in protein-protein interactions, quenching may occur. Quenching results the decrease of the fluorescent light. Are fluorophore and quencher are now separated quenching will no longer occur and the fluorescence signal emits a can be detected.

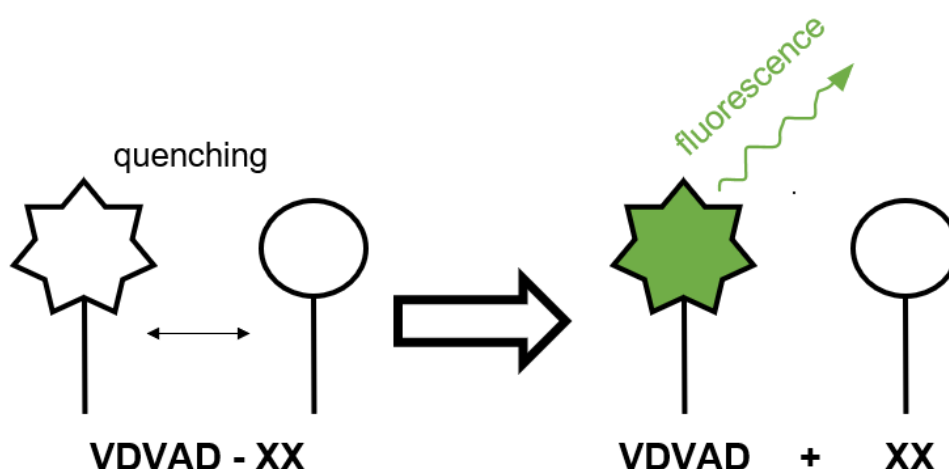

Figure 4: FRET mechanism; VDVAD represents the specific cleavage site of cpCaps2. XX means any amino acids, since caspase 2 does not need any specific amino acid C-terminal of its cleavage site

When using this principle now to detect enzymatic activity one needs a substrate with one part being a chromophore and the other part being close enough to quench the fluorescent light. Additionally, the substrate must be specific for the enzyme to be detected. If the enzyme is added to the substrate, it starts to cleave at the cleavage site and thus separates the chromophore from the remaining substrate leading to a fluorescent signal. The signal intensity can then be linked to the enzymatic activity in the sample.

2 Objectives

This work focuses on the purification process of cpCasp2, shown in Figure 3A, where the harvested *E.coli* cells were disrupted by high pressure homogenization, followed by clarification using centrifugation and filtration and subsequently purified using IMAC as a capture step. After IMAC the buffer was exchanged using diafiltration followed by a polishing step using cation exchange chromatography.

The primary aim of this work was to optimize the existing process to increase the protein recovery without sacrificing purity.

As a first step the individual yields as well as the overall process yield of the existing process were to be determined. The major challenge hereby was to find an analytical method to quantify the enzymatic activity especially in crude samples such as homogenate and flowthrough or wash fraction of IMAC capture. The assay had to be optimized to minimize matrix effects of crude samples. Further it had to be adapted to function as a fast and easy to handle routine assay.

With a functioning analytical method, the initial process now had to be characterized determining the step yields. This serves as a basis to decide which of the individual unit operations are addressed in the following optimization procedure.

The unit operations, which now were subject to optimization were now performed with varying process parameters to find the optimal conditions. The optimization goal hereby was the increase in protein recovery.

As an additional optimization approach, the buffer exchange with subsequent cation exchange chromatography should be substituted by a mixed mode chromatography. The elution of the IMAC capture could be directly loaded onto the mixed mode column and hence buffer exchange is no longer necessary. This would reduce the whole process by one unit operation.

3 Materials & Methods

3.1 FRET assay

3.1.1 Optimized assay for enzyme quantification

Quantification of caspase samples in terms of enzymatic activity was carried out using FRET assay. The substrate with the structure Abz-VDVAD-GA-Dap-(Dnp) (Bachem AG, UK) was dissolved in substrate buffer (10 mM HEPES, pH 7.5) to aim a concentration of 750µM and aliquots were frozen at -20 °C. Assay buffer (50mM Hepes, 150 mM NaCl, 50 mM EDTA, 0.025% TWEEN 80, pH 6.6) for dilution of samples was prepared freshly from stock solutions and degassed before use. As an internal standard highly purified cpCasp2 was used to allow quantification of caspase samples. Samples were diluted in a black 96-well-plate (Greiner AG, Austria). Afterwards substrate was added to yield a concentration of 100 µM substrate in each well. Immediate incubation at 37 °C for 15 min at 450 rpm using Thermomixer (Eppendorf AG, Germany) was started. Reaction was stopped with a final concentration of 2.2 mM cystamine per well as soon as the incubation time was finished. Fluorescence measurement was done in multiple reads per well with excitation at 320 nm and an emission at 420 nm using Tecan Infinite M200 Pro plate reader (Tecan Trading AG, Switzerland). An internal standard of highly purified caspase was used for determination the enzymatic activity.

3.1.2 Optimization of FRET assay

In order to prevent matrix effects, DTT as well as EDTA were added to the assay buffer. For EDTA addition, 8 samples summarized in Table 1 were prepared.

Table 1: Samples tested to investigate the influence of EDTA

Samples
Assay buffer
Assay buffer + EDTA
Purified cpCasp2
Purified cpCasp2 + EDTA
E. coli lysate
E. coli lysate + EDTA
E. coli lysate + purified cpCasp2
E. coli lysate + purified cpCasp2 + EDTA

Assay buffer consisted of 50 mM MES, 150 mM NaCl, 0.025% Tween 80, pH 6.6. The *E.coli* lysate was of strain BL21 DE3 producing β-galactosidase. Purified cpCasp2 was present in a concentration of 4.4 mg/mL in formulation buffer (50 mM citrate, 1M NaCl, pH 5.0) and diluted to a concentration of 0.5 mg/mL per sample. EDTA was added to yield a concentration of 50 mM per sample.

All samples were filled into wells of black 96-well plate (Greiner AG, Austria). Substrate was added to yield a concentration of 100 µM per well. The samples were incubated at 37 °C for 15 min at 450 rpm using a Thermomixer (Eppendorf AG, Germany) and stopped afterwards with cystamine with a final concentration of 2.2 mM. Fluorescence was then measured in multiple reads per well with excitation at 320 nm and an emission at 420 nm using Tecan plate reader (Tecan Trading AG, Switzerland).

For DTT addition the same 8 samples were used but instead of EDTA, DTT was added to a final concentration of 10 mM per well.

Table 2: Samples tested to investigate the influence of DTT

Samples
Assay buffer
Assay buffer + DTT
Purified cpCasp2
Purified cpCasp2 + DTT
E. coli lysate
E. coli lysate + DTT
E. coli lysate + purified cpCasp2
E. coli lysate + purified cpCasp2 + DTT

The assay buffer in this case already contained EDTA giving a composition of 50 mM MES, 150 mM NaCl, 50mM EDTA, 0.025% Tween 80 and pH 6.6. As for the EDTA trial, the samples were incubated, the reaction was stopped, and the fluorescence measured.

3.1.3 Standard addition method

Homogenate (1000 bar, 2 passages) of cpCasp2 was spiked with purified cpCasp2 to quantify crude samples. Purified caspase with a concentration of 2.29 mg/mL was diluted to 1.0, 0.5 and 0.1 mg/mL. Assay buffer was added to rows B-H of a black 96-well-plate (Greiner AG, Austria). In row A the homogenate was added and then a serial dilution was performed until row G. Row H was assay buffer only, serving as blank. In triplicates assay buffer (column 1-3), 1 mg/mL (column 4-6), 0.5 mg/mL (column 7-9) and 0.1 mg/mL (column 10-12) was added to the homogenate samples. Afterwards the samples were incubated for 15 min at 37 °C at 450 rpm using Thermomixer (Eppendorf AG, Germany). The reaction was stopped with cystamine with a final concentration of 2.2 mM. Fluorescence was then measured in multiple reads per well with excitation at 320 nm and an emission at 420 nm using Tecan Infinite M200 Pro plate reader (Tecan Trading AG, Switzerland).

3.2 High pressure homogenization

Prior to homogenization, the *E. coli* cells were separated from culture media by centrifugation using Avanti JXN-26 centrifuge (Beckman Coulter Life Sciences, USA) at 18,600 rcf for 15 min at 4 °C.

200 g of cell wet mass were solubilized in 1 L of homogenization buffer (50 mM NaH_2PO_4, 150 mM NaCl, pH 7.0) resulting in a concentration of around 170 g/L. Solubilisation was carried out at 4 °C by constant stirring. To optimize the yield of cell disruption, using Panda PLUS 2000 (GEA, Germany), following factors were included in the factorial design plan:

- homogenization pressure [bar]: 700, 850, 1000, 1200, 1400, 1600
- number of passages [-]: 1, 2

After high pressure homogenization the suspension was centrifuged at 18,600 g for 2 h at 4 °C using Avanti JXN-26 centrifuge. Supernatant was stored at -80 °C.

For the following IMAC capture experiments, 300 g/L *E. coli* cells were solubilized in homogenization buffer (50 mM NaH_2PO_4, 150 mM NaCl) with pH 7.0 as well as with pH 8.0. High pressure homogenization was carried out at 1400 bar in 2 passages.

3.3 IMAC Capture

All chromatographic experiments were carried out using ÄKTA Pure 25 (GE Healthcare, Sweden) chromatographic system and Unicorn 4.1 SP2 software (GE Healthcare, Sweden). The various stationary phases were packed into Tricon columns (GE Healthcare, Sweden) with an inner diameter of 5 mm or 10 mm and varying lengths. Tricon 10/50 was used for the experiments testing the influence of ligand and immobilized metal. Tricon 5/100 was used for the other experiments performed. Sample loading was performed using a 10 mL Superloop (GE Healthcare, Sweden). Protein was detected at a wavelength of 280 nm as well as 320 nm. Used columns were sanitized for 20 min with 1 M NaOH and stored in 20% ethanol. All caspase samples were kept at 4 °C and aliquots were frozen at -80 °C.

3.3.1 Screening for loading conditions

In total 12 different buffer conditions were tested. The equilibration buffer was varied in the concentrations of imidazole and NaCl and pH values.

- NaCl concentration [mM]: 150, 300
- imidazole concentration [mM]: 20, 25, 30
- pH: 7.0, 8.0

The buffering compound of the equilibrium and elution buffers 50 mM NaH_2PO_4 was in all conditions.

The elution buffer contained 500 mM imidazole but the NaCl concentrations and pH values were varied to match the corresponding equilibration buffer.

Before loading, the protein solution was adjusted to the according imidazole and NaCl concentration and filtered (0.22 µm). 25-30 mg of caspase was loaded for each experiment. The flowrate was set to achieve a residence time of 3 min. Loading condition screening tests were carried out using NTA tagged IMAC Sepharose FF (GE Healthcare, Sweden) with nickel as immobilized metal.

Table 3 gives an overview of the method used for caspase purification trials.

Table 3: Method for caspase purification

Step	Volume [CV]	Buffer
Equilibration	5	100 % equilibration buffer
Sample load	-	-
Wash	10	100 % equilibration buffer
Elution	10	100 % elution buffer

One fraction of each, flow-through, wash and elution, were collected in 15 mL centrifugation tubes (Greiner AG, Austria) and immediately stored at 4 °C. Aliquots were taken and stored at -80 °C for later use. Caspase content was quantified using FRET-assay, with pooled flow-through and wash fraction. The eluate fraction was also quantified using RP-HPLC analysis.

3.3.2 Test for solubility issue

To test whether precipitation occurred on the column due to low cpCasp2 solubility, caspase was loaded in varying concentrations on the stationary phase (namely, 10, 15 and 25 mg/mL). The lysate in homogenisation buffer (50 mM NaH_2PO_4, 150 mM NaCl, pH 8.0), was adjusted to a salt concentration of 300 mM NaCl and an imidazole concentration of 20 mM and filtered (0.22 µm) before loading. The purification followed the method in Table 3 with a residence time of 3 min. Buffers used:

- Equilibration buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0)
- Elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0)

The eluates were quantified using RP-HPLC analysis.

3.3.3 Influence of ligand and immobilized metal

The influence of ligand and immobilized metal was tested with WorkBeads resins 40 NTA and 40 IDA (Bio-Works, Sweden). Four different metals (zinc, copper, nickel, cobalt) were immobilized on each of the two resins resulting in 8 different combinations. The resin was charged with the metals by loading 2 CV of 50 mM metal salt ($ZnCl_2$, $Co(II)SO_4$, $Cu(II)SO_4$, $Ni(II)SO_4$)

Caspase purification using the charged WorkBeads columns was carried out as shown in Table 3 with a residence time of 1 min. 8 mg/mL of caspase was loaded onto each column. The lysate (pH 8.0) was adjusted to 300 mM NaCl and 10 mM imidazole and filtered (0.22 µm) before loading. Buffers used:

- Equilibration buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0)
- Elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0)

Increasing imidazole concentrations (20 mM, 25 mM, 30 mM) during loading were tested on both zinc and also the nickel WorkBeads stationary phase. Purification experiments were performed according to the method in Table 3 with a residence time of 3 min for IMAC Sepharose FF and 1 min for WorkBeads 40 NTA. 8 mg/mL of caspase were loaded and the lysate was adjusted to 300 mM NaCl, the according imidazole concentration and filtered (0.22 µm) before loading.

All eluates were quantified and purity was calculated using RP-HPLC analysis.

3.3.4 Additives

Tween 80 and DTT were tested to influence the binding behaviour of cpCasp2. Following conditions were tested with IMAC Sepharose FF (GE Healthcare, Sweden) with immobilized nickel ions:

- Tween 80: 0.025% (v/v)
- DTT [mM]: 10

The conditions were not tested in combinations giving a total of 5 different conditions to be tested. Both equilibration (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole,

pH 8.0) and elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0) as well as the lysate were supplemented with the according additive before purification. Purification was carried out according to Table 3 with a residence time of 3 min.

All eluates were quantified and purity was calculated using RP-HPLC analysis.

3.4 Mixed mode chromatography

Mixed mode chromatography was tested to replace the buffer exchange and polishing chromatography step after IMAC capture. For this purpose, a BabyBio 40 TREN 1 mL prepacked column (Bio-Works, Sweden) was used. The IMAC capture eluate, present in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0) was loaded directly onto the mixed mode column. 2.55 mg/mL of caspase were loaded. The purification was performed according to the method shown in Table 4 with a residence time of 1 min during non-loading steps and 2 min during loading.

Table 4: Method for caspase purification using mixed mode chromatography

Step	Volume [CV]	Buffer
Equilibration	5	100 % equilibration buffer
Sample load	-	-
Wash	10	100 % equilibration buffer
Elution	10	Linear Gradient: 0-100 % elution buffer

- Equilibration buffer (50 mM NaH_2PO_4, 300 mM NaCl, 500 mM imidazole, pH 8.0)
- Elution buffer (50 mM NaH_2PO_4, 1 M NaCl, pH 8.0)

Flow-through, wash and eluate were quantified using RP-HPLC analysis.

3.5 Analytical methods

3.5.1 SDS-PAGE

Protein samples were diluted to a concentration of about 0.5 mg/mL. 13 µL of sample were mixed with 5 µL NuPAGE LDS sample buffer (ThermoFisher Scientific Inc., USA) and 2 µL of 2 M DTT, as reducing agent. Afterwards the samples were incubated for 10 min at 70 °C in the Thermomixer (Eppendorf AG, Germany) to completely denature the proteins. As a protein standard, SeeBlue Plus2 pre-stained protein standard (ThermoFisher Scientific Inc., USA) was used. 15 µL of samples were loaded onto the polyacrylamide gel (4-12% Bis-Tris Gel) (ThermoFisher Scientific Inc., USA) and run for 50 min at 200 V and 400 mA in 1x MES running buffer.

All solutions which were used for SDS-PAGE staining are listed in Table 5. The gel was first put into the fixing solution for at least 25 min. After fixing the gel it was put in water for around 10 min and afterwards stained with Coomassie Blue for approximately 30 min. The gel was then de-stained until the protein bands were visible.

The gel was scanned using Epson Perfection V800 Photo (Epson, USA) scanner.

Solution	Components
Fixing solution	50 % (v/v) Ethanol (96%), 10 % (v/v) Acetic Acid (100%), water
Staining solution	1.16 g Coomassie Blue R250, 25% (v/v) Ethanol (96%), 8% (v/v) Acetic Acid (100%), water
De-staining solution	25% (v/v) Ethanol (96%), 8% (v/v) Acetic Acid (100%), water

3.5.2 RP-HPLC

Quantification of cpCasp2 in pure samples was carried out using RP-HPLC. The analysis was performed on the Waters e2695 HPLC (Waters, UK) using a TSKgel Protein C4-300 (L × I.D. 5 cm × 4.6 mm, 3 µm) column with a guard column (Tosoh, Japan). UV absorption was measured at 214 nm.

The flowrate was 1 mL/min.

The column was kept at a temperature of 40 °C ± 3 °C.

Buffer A and B were the following:

- Buffer A: HQ-H2O + 0.15% TFA
- Buffer B: Acetonitrile+ 0.15% TFA

The method used is shown in Table 5.

Table 5: Method for caspase analysis by RP-HPLC

Time [min]	Buffer A [%]	Buffer B [%]
0	98	2
1	98	2
2	75	25
8	50	50
15	45	55
16	10	90
18	10	90
18.01	98	2
19	98	2

To accommodate background signals, a buffer blank was subtracted from each run. All samples were supplemented with DTT to a final concentration of 100 mM.

Quantification of cpCasp2 was performed by using a calibration curve. Purified cpCasp2 was diluted to three different concentrations (0.5, 1 and 2 mg/mL) in triplicates and analysed. Figure 5 shows the generated calibration curve.

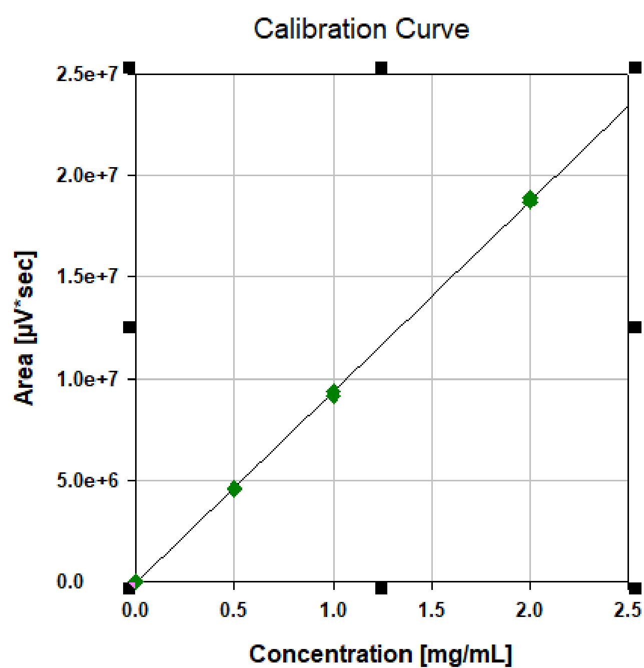

Figure 5: Calibration curve for cpCasp2 quantification, R^2 *= 0.9998*

4 Results

4.1 FRET-assay

In order to accurately determine the yields of the individual unit operations, a sensitive and specific analytical method, especially for crude samples, is required. A FRET-assay was developed to function as an analytical assay for the quantification of caspase-2 variants. By measuring the enzymatic activity, the process can be optimized for the most important quality attribute of the target enzyme. Furthermore, an internal standard of purified caspase the target enzyme concentration can be determined, assuming constant specific activity throughput the downstream process.

Using purified caspase, the results of the assay show a linear behaviour, which is shown Figure 6. As long the enzyme is present in a pure form, the assay works reliably and the enzymatic activity and hence also the concentration of the caspase can be determined.

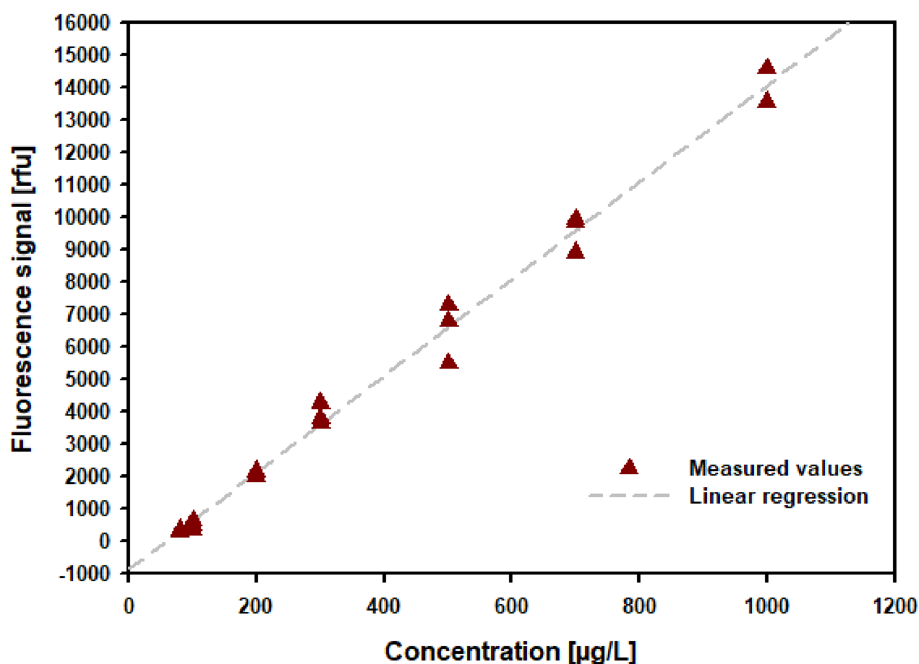

Figure 6: Linearity of FRET-assay in purified samples

Crude samples in particular are difficult to accurately quantify, due to the complicated matrix. One potential matrix effect are host cell proteases, which also cleave the substrate resulting in fluorescence signal increase in the absence of caspase. An in silico digest of the FRET substrate, with the sequence VDVAD/GA, was performed by using the online peptide cutter from ExPASy [29]. The only enzyme that is present in *E. coli* and able to cleave the same sequence as caspase-2 is a metalloprotease aminopeptidase N. Inhibition of this enzyme was attempted with the addition of EDTA, since Zn2+ ions are a necessary co-factor for this protease [30].

To test for the presence of a metalloprotease activity, FRET samples were measured with and without EDTA. Results of this approach are seen in Figure 7.

As a negative control assay buffer was used, giving a fluorescence signal corresponding to the substrate only. It does not change in the presence of EDTA.

Cell lysate of *E. coli* cells expressing the non-caspase model protein β-galactosidase, was used to investigate the effect of EDTA on the metalloprotease aminopeptidase N [31]. Since the same *E. coli* strain BL21 DE3 is used in production of cpCasp2 the host cell proteins should be similar. The lysate samples showed different fluorescence increases. Without EDTA (Figure 7, yellow bars) the fluorescence signal increased slowly over time. When EDTA was present (green bars), the fluorescence signal resembles that of the negative controls (red and orange bars). This strongly suggests that a host cell enzyme able to cleave the used substrate is present. The contribution of this enzyme to the matrix effects however is rather small and does not explain them completely.

As expected, purified caspase cleaves the substrate effectively yielding higher fluorescence. What is not clear in this case is why only purified caspase is influenced positively by EDTA. A possible explanation may be that metal ions inhibit or negatively influence the activity of the caspase and EDTA addition therefore leads to an increase in activity. This may suggest that the cleaving mechanism of purified caspase is somehow slightly enhanced in the surrounding of a chelating agent.

As soon as the caspase is present in a matrix the effect is reverse. The decrease of fluorescence signal in this case may also be partly due to the inhibition of the

metalloprotease of the lysate. What was rather surprising was the almost three-fold higher fluorescence signal when testing a mixture of cell lysate and purified caspase. What remains unclear is why this large matrix effects only occur with lysate in combination with caspase and not with lysate only.

Although aminopeptidase N was not the main reason for matrix effects it was decided that EDTA will be used as part of the assay buffer for any further experiments, since it decreases the net matrix effect that we observed from 5-fold to 2.5-fold.

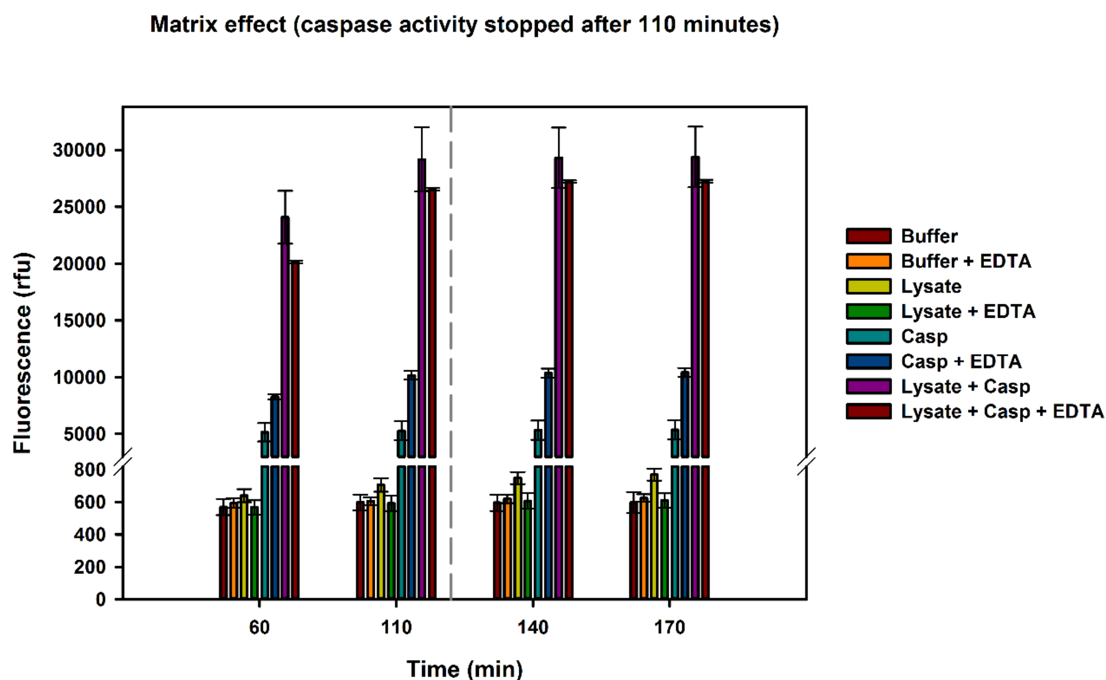

Figure 7: Influence of EDTA on matrix effects in FRET assay. The fluorescence was measured in several time intervals. The dashed line represents deactivation of caspase activity by addition of cystamine

It was hypothesized that the crude sample may provide a reduced environment in which the activity of the caspase is enhanced. Therefore, DTT was added to any sample to yield a fully reduced redox potential in each case to see whether this effect is the reason for the higher enzymatic activity in the crude samples.

Results are shown in Figure 8. The assay buffer used already contained EDTA. The differences in absolute fluorescence between the first trial testing the influence of EDTA (Figure 7) and the one with additional testing of DTT are due to different incubation times. Stopping of the enzymatic reactions by addition of cystamine did not work in the presence of DTT since cystamine also is reduced and hence can no

longer inhibit the enzymatic activity of cpCasp2. Therefore, fluorescence is further increasing even though the reaction should have been stopped. There was no visible effect of DTT when using assay buffer or lysate (red, orange, yellow and green bars). Addition of DTT to purified caspase gives a slightly higher signal (blue bars). Other experiments (data not shown) led to the conclusion that DTT may contribute to the activity of caspase by changing its redox potential. This may also be the explanation why DTT addition to the sample containing lysate and purified caspase (dark red bars), further increases the fluorescence by 1.5- to 2-fold compared to the same sample without DTT. Next to the fact that DTT interferes with the stopping of the reactions, there was no improvement of the assay in terms of matrix effect and therefore no DTT will be added to the assay in further experiments.

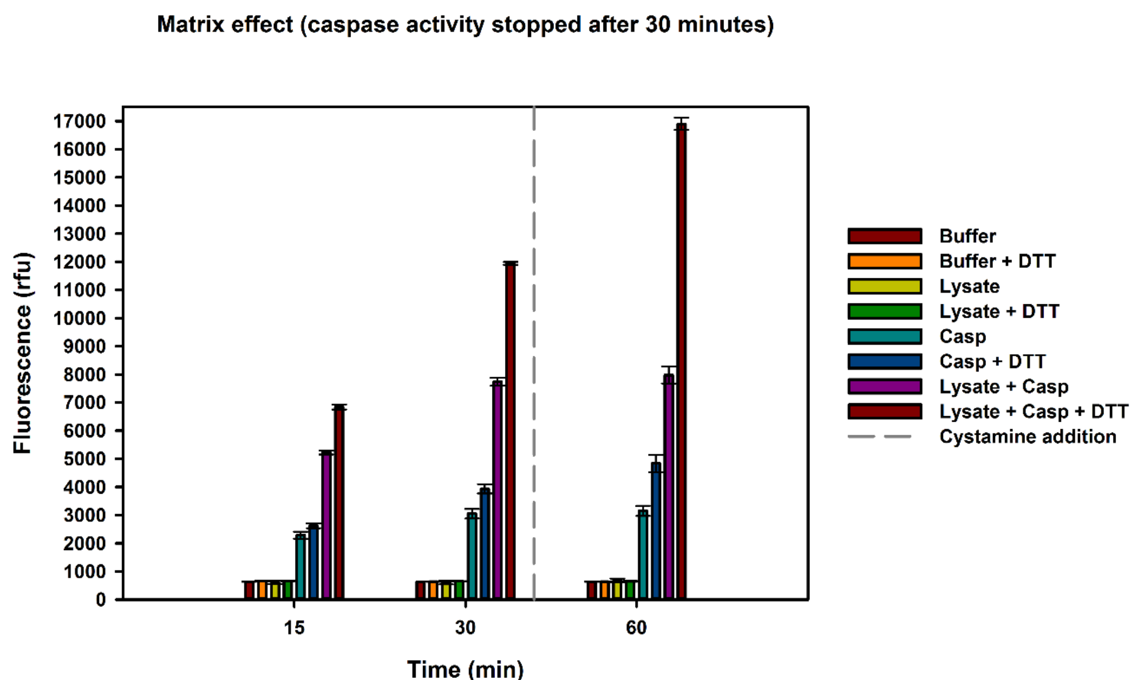

Figure 8: Influence of DTT leading to a changed redox potential. The dashed line represents deactivation of caspase activity by addition of cystamine. All samples contained 50mM EDTA

Summarizing the results of the assay development it turned out that it works as a reliable method for purified samples but is influenced highly by the matrix if the cpCasp2 is not pure. Therefore, reasons for these matrix effects were investigated. One minor effect was caused by a metalloprotease, which could be inhibited by EDTA. The hypothesis that a change in redox potential may have beneficial effects was tested by DTT addition but failed to have a positive effect on the assay. Since

none of the tested substances led to a significant reduction of matrix effects it was decided to analyse crude samples using a standard addition method where known concentrations of purified cpCasp2 were added to the samples to correct for matrix effects.

4.2 Process Characterization

Development and optimization of the FRET assay gave rise to the possibility to specifically determine enzymatic activity of cpCasp2 especially in crude samples. The initial purification process prior to optimization could be characterized and the individual step yields were calculated (Figure 9: Process scheme of initial cpCasp2 purification process prior to optimization with step and overall yield (yellow boxes) The two unit operation having the lowest step yields were identified to be cell disruption and clarification including high pressure homogenization, with a step yield of 61%, and IMAC Capture as the second unit operation with a high optimization potential and a step yield of 43%. Hence these were chosen to be the focus of the optimization approach in this work. The subsequent buffer exchange using diafiltration and cation-exchange chromatography already had an acceptable yield and hence were not optimized.

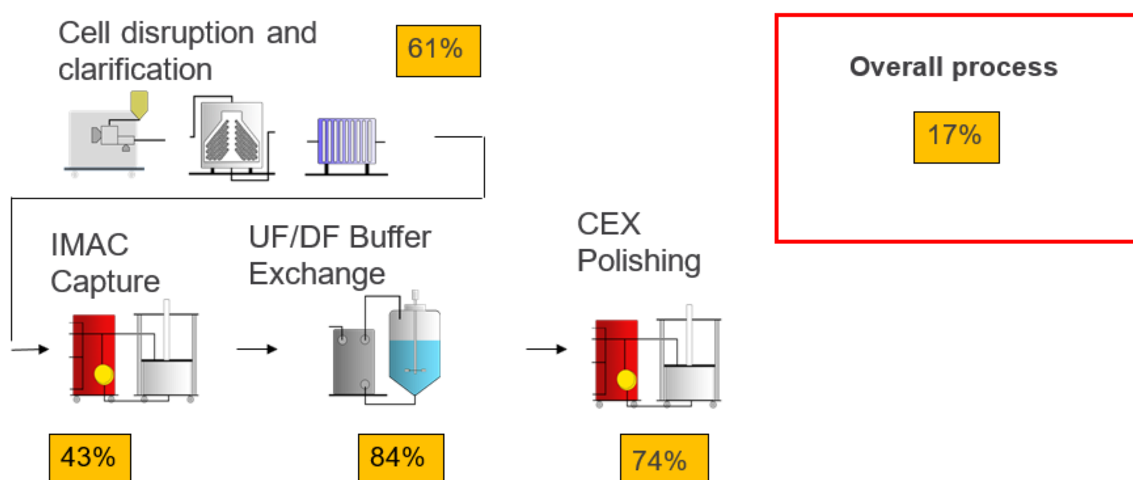

Figure 9: Process scheme of initial cpCasp2 purification process prior to optimization with step and overall yield (yellow boxes)

4.3 High pressure homogenization

In this work, cell disruption efficiency of different pressures after one and two passages was evaluated by measuring the enzymatic activity of caspase variant cpCasp2 using FRET-assay with standard addition. This method was chosen since it is not possible to quantify the crude samples using a reference standard of purified caspase due to matrix effects. Therefore, the samples of 1000 bar and 2 passages were spiked with cpCasp2 in three different known concentrations. The results of the other pressures and passages were evaluated relative to these samples.

The yield, shown in Figure 10, is increasing with increasing pressure until a maximum of 97% at 1400 bar. This indicates that the *E. coli* cells from strain BL21 DE3 are fairly hard to break up, since usually pressures around 700 bar are enough to reach nearly complete disruption [32]. With further increase of pressure, the measured enzyme activity decreased again. It can be assumed that at 1600 bar the same amount of protein was released from the cell as with 1400 bar, however the activity may be reduced due to damage of the protein. This damage is probably caused by the high energy input leading to protein denaturation. Testing the effect of the number of passages showed that there is indeed an increased activity and therefore most probably also an increased cell disruption when performing two passages instead of one. This may also support the hypothesis that the cells are very robust and high power input is needed to break them.

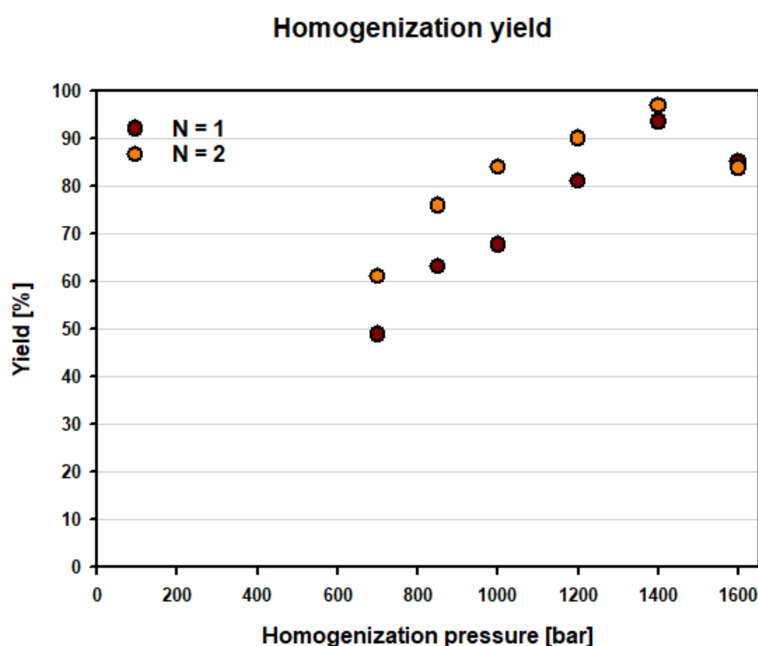

Figure 10: Disruption yield of high-pressure homogenization depending on pressure and number of passages (N).

Development of the FRET-assay made it possible to quantify the enzymatic activity of cpCasp2 in crude sample as lysate. As shown in the results of FRET-assay optimization (see Figure 7) cpCasp2 activity is strongly increased by the surrounding matrix in the lysate. Therefore, a standard addition method was used to determine the absolute enzymatic activity in the samples. However even with this there may still be some inaccuracies in the results especially because the reference for yield calculation was cell disruption by enzymatic cleavage and SDS-PAGE quantification, which is known to be rather inaccurate. Even though it cannot be precisely stated that the best performing conditions yield exactly 97%, it is still valid that a pressure of 1400 bar results in much higher cell lysis than 700 bar.

An overview of the optimized conditions for high pressure homogenization is shown in Table 6.

Table 6: Overview of optimized homogenization conditions

Pressure [bar]	1400
Passages	2

4.4 IMAC Capture

The following purification process, was an IMAC capture step having a yield of around 43% performing this unit operation using the current conditions (50 mM Na-phosphate, 20 mM imidazole, 500 mM NaCl, pH 7). Several conditions of pH, NaCl and imidazole were tested to increase the yield and find out why the performance was rather low before the optimization approach. Na-phosphate was part of each tested buffer and was not changed, since it is recommended in IMAC buffers [14].

4.4.1 Screening for loading conditions

Different loading conditions with varying imidazole and salt concentrations as well as different pH values were tested. For the pH it was expected that the influence is protein specific rather than process specific since pH influences the protonation state of the amino acids and therefore the net charge of the protein. The closer the pH comes to the isoelectric point, the less soluble a protein gets. Salt concentration can have an influence on protein stability [33]. A higher salt concentration may increase the yield either by stabilizing the protein or by decreasing the binding interactions, both leading to decreased irreversible column binding. It is also possible that the higher ionic strength reduces the binding events of charged impurities. During adsorption low concentrations of imidazole are used to provide competitive binding for Ni-ions to lower the affinity for impurities and therefore increasing the purity of the eluted target protein. Too high concentrations however will also lead to flow through of the his-tagged target protein.

Therefore, three different imidazole concentrations were tested to investigate the optimal concentration where the purity is the highest and the loss of cpCasp2 in the flow-through the lowest. Additionally, pH and salt concentrations were also tested in two different values each.

Figure 11 shows the protein amount that could be recovered during elution in percentage of total protein loaded. As shown a salt concentration of 300 mM NaCl with a pH value of 8.0 turned out to be the best performing condition.

The effect of a higher yield at the higher pH value results from the fact that proteins have a lower solubility closer to the isoelectric point. With a pI of approximately 6.5 cpCasp2 is more soluble at pH 8.0 and therefore more of the loaded protein could have been recovered. As expected the higher salt concentration led to an increased recovery probably by stabilizing the protein or by decreasing the binding interactions. It is also possible that the higher ionic strength reduces the binding events of charged impurities.

Concerning the concentration of imidazole during loading, it seems that there is no significant influence regarding recovery. This is confirmed by the fact that only very little of the loaded caspase could be found in the flow-through and wash fraction (see Figure 12 orange bars). In this case however, the imidazole concentration did not influence the purity in a significant manner. Independency of yield from imidazole concentration provides a more robust process in this case since inaccurate addition of imidazole before loading does not result in protein loss. A concentration of 20 mM imidazole will be used in further trials. Summing up the conditions chosen to be the best performing, they are 300 mM NaCl, 20 mM imidazole and a pH of 8.0 increasing the yield from 43% to 63% compared to the conditions used prior to the screening.

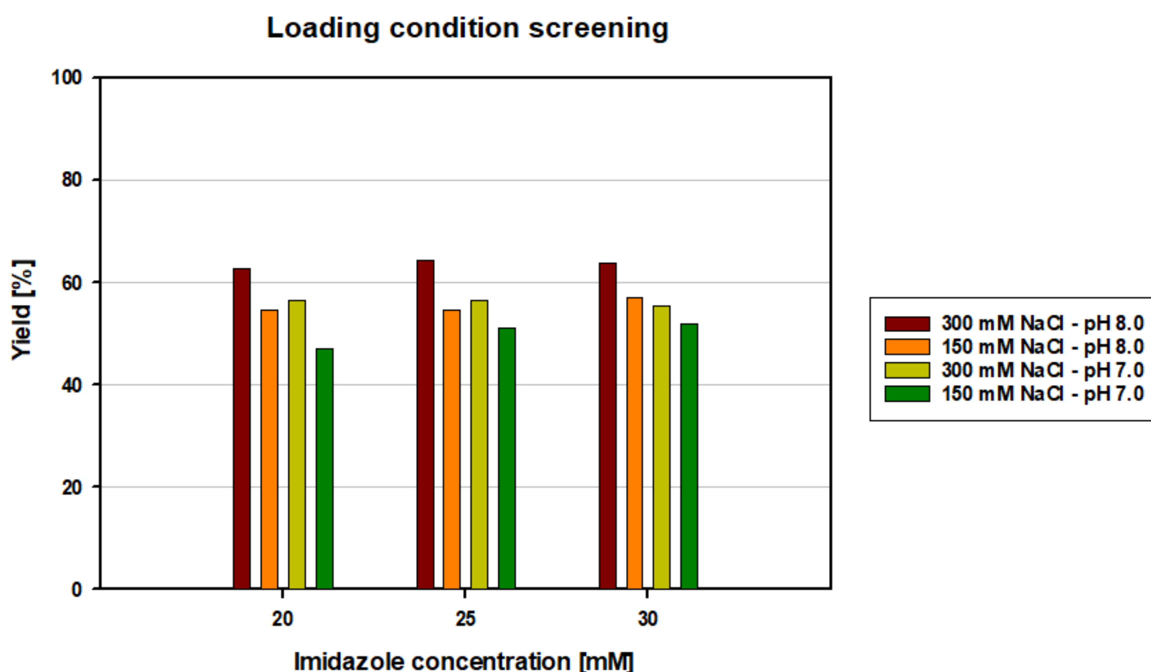

Figure 11: Influence of pH, salt and imidazole concentration on the yield of cpCasp2 purification in IMAC capture. Every buffer also contained 50 mM Na-phosphate.

4.4.2 Closing the mass balance

Even though the yield could be increased to more than 60%, still nearly 40% of protein is not showing up in the mass balance (see Figure 12).

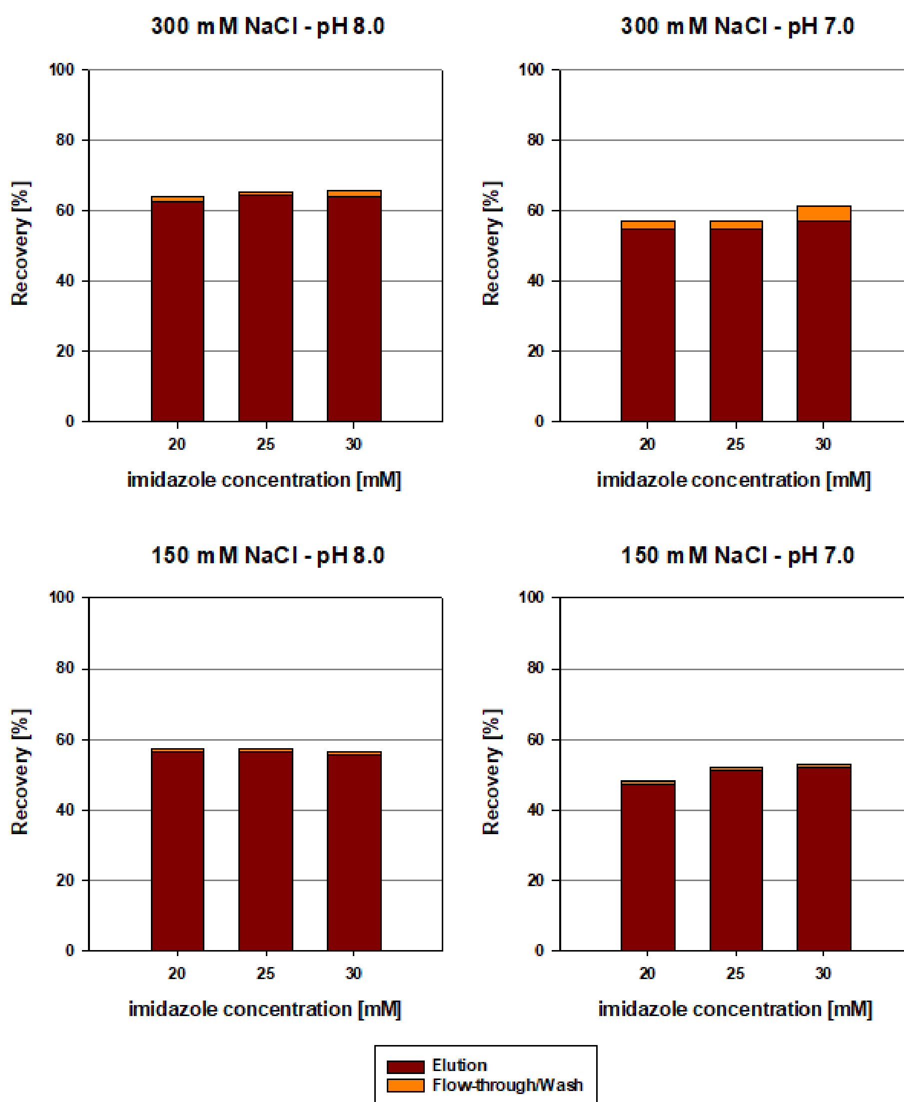

Figure 12: Mass balance of the IMAC capture using varying pH and salt concentration

To find out whether the 40% of protein are maybe still bound to the column, purified caspase as well as homogenate were loaded and eluted with 500 mM imidazole. The used columns were unpacked directly after elution and the used resin was incubated in 10% SDS solution and an SDS-PAGE was performed.

The SDS-PAGE gel (shown in Figure 13) clearly shows that a high amount of caspase was still bound to the stationary phase indicated by the thick bands

between 28 and 33 kDa being cpCasp2 with a molecular weight of approximately 35 kDa. In lane 1 the protein standard was loaded. Lanes 2 and 3 show purified caspase as a reference to also justify the statement of the think bands being cCASP2. Lanes 4-6 and 7-12 show the proteins that is still bound to the stationary phases, which was loaded with purified caspase only. In lanes 4-6 one can see the proteins that remained on the stationary phase loaded with clarified homogenate. No matter if the caspase loaded was in high purity or still in a rather crude solution, a high percentage of it remained onto the column and could not be recovered.

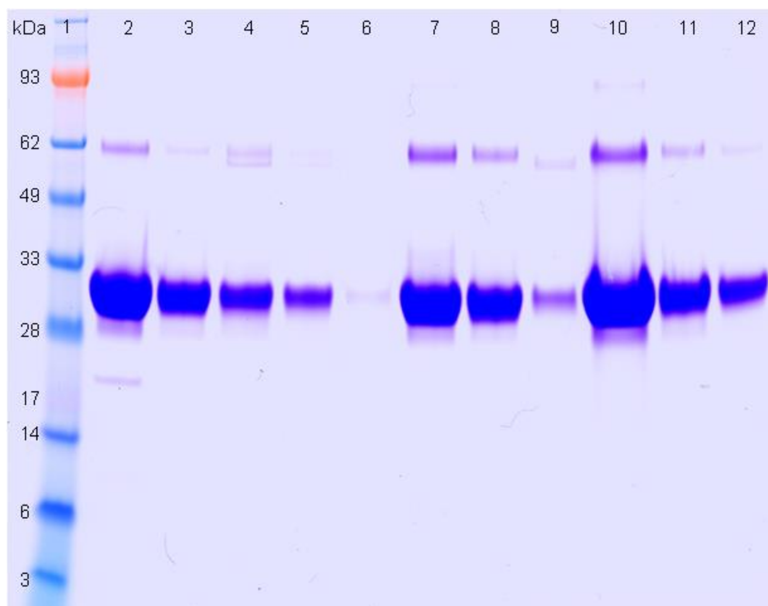

Figure 13: SDS-PAGE of unpacked stationary phases of purification of crude samples and already purified cpCasp2; 1… protein standard SeeBlue Plus2, 2-3… purified caspase reference standard (5x, 10x diluted), 4-6… cell lysate loaded (5x, 10x, 100x diluted), 7-12… purified cpCasp2 loaded (5x, 10x, 100x, 1x, 5x, 10x diluted)

4.4.3 Test for solubility issue

To investigate the reason for this tight binding and the resulting inability for elution of cpCasp2 from the stationary phase several hypotheses were tested.

First is was hypothesized that cpCasp2 is rather insoluble at high concentrations and thus may precipitate during elution due to a temporary high concentration of the protein. Different amounts of caspase were loaded onto the column. According to the hypothesis of a present solubility issue, the lowest amount of caspase loaded should yield in a lower concentration of protein during elution and hence to a nearly

complete recovery. This however was not the case. As seen in Figure 14, the recovery of caspase during elution was independent of the amount loaded and again only around 60-65% from the loaded protein could be recovered. This indicates that there is probably no precipitation of caspase due to a too high temporary protein concentration during elution.

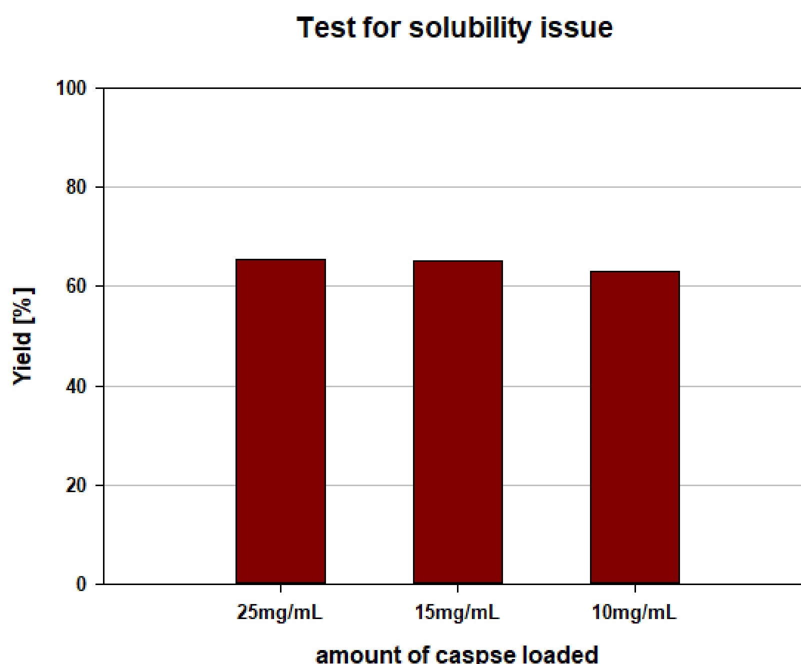

Figure 14: Influence of different amounts of protein loaded on the yield of cpCasp2 to test for a possible solubility issue. The loaded protein was present in clarified homogenate.

Local high concentrations of proteins binding to the resin beads cannot be avoided since with pore diffusion as the driving force, the shrinking core model [34] applies. In this model with a high affinity of the proteins for the immobilized ligand, the proteins use the nearest binding sites and do not diffuse further into the pores. Hence, regardless on the amount of protein loaded, there will be in any case a high local concentration approaching the equilibrium binding capacity. Thus, the solubility of the protein was most probably exceeded in any of the tested conditions.

4.4.4 Influence of ligand and immobilized metal

Another hypothesis claimed the tight binding of caspase to the stationary phase to be a result of too strong interaction with the Nickel-ion or the coupling of the metal ion to the backbone using NTA. Thus, four different metal ions as well as two

different coupling mechanisms, namely NTA and IDA were tested. An overview of these regarding yield and purity can be seen in Figure 15.

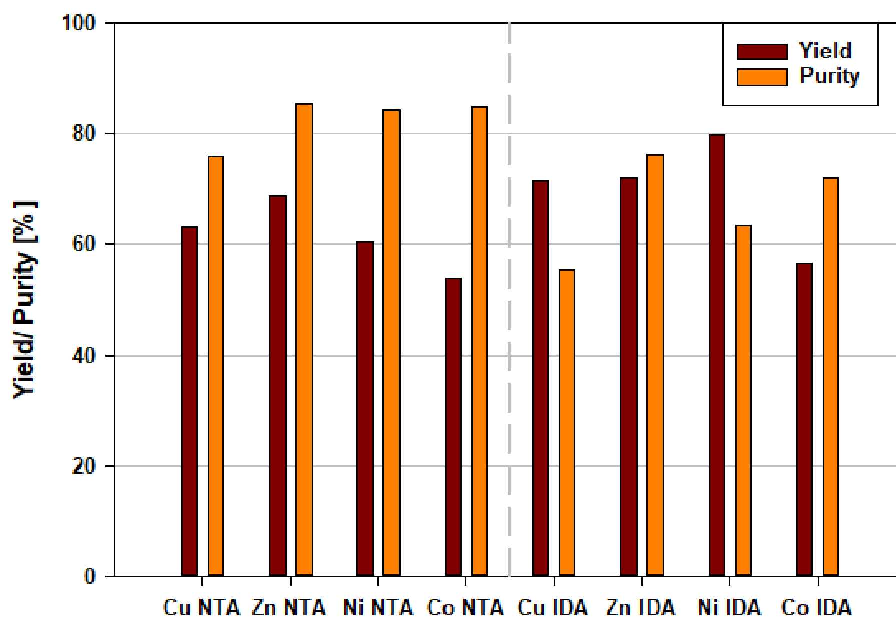

Figure 15: WorkBeads 40 NTA and 40 IDA loaded with different metal ions to investigate yield and purity of cpCasp2

Comparing the different ion coupling mechanisms it is obvious that they behave inversely in both yield as well as purity. Whereas the NTA coupled ions achieve a purity of 75% up to 85% the yield is rather low with a maximum of 69% with zinc coupled. IDA as a coupling mechanism in contrast yielded higher yields of 80% with nickel coupled, but the purity is unsatisfactory in all samples with only zinc higher than 75%. Comparing NTA and IDA coupling in terms of recovery, where also the losses during loading and wash are taken in consideration, there are major differences. The WorkBeads NTA coupling behaves similar to the IMAC Sepharose, which also uses NTA to couple the ions. There only very little amounts, lower than 0.2%, of caspase are not binding and leave the column as flow-through or during the wash (see Figure 16). When trying to capture the caspase with IDA linked ions, there are much higher losses during loading with percentages from 5% for copper to up to 25% for zinc. This may be due to the fact that the affinity of the ions and the proteins are not that strongly when coupled with IDA and thus a much higher percentage is not retained. There may also be the possibility that the column was

overloaded but this is rather unlikely since with only 8 mg protein / mL of resin was loaded which does not exceed the dynamic binding capacity of more than 60 mg/mL [24]. The reduced residence time when operating with WorkBeads may also lead to an increase in protein losses during loading.

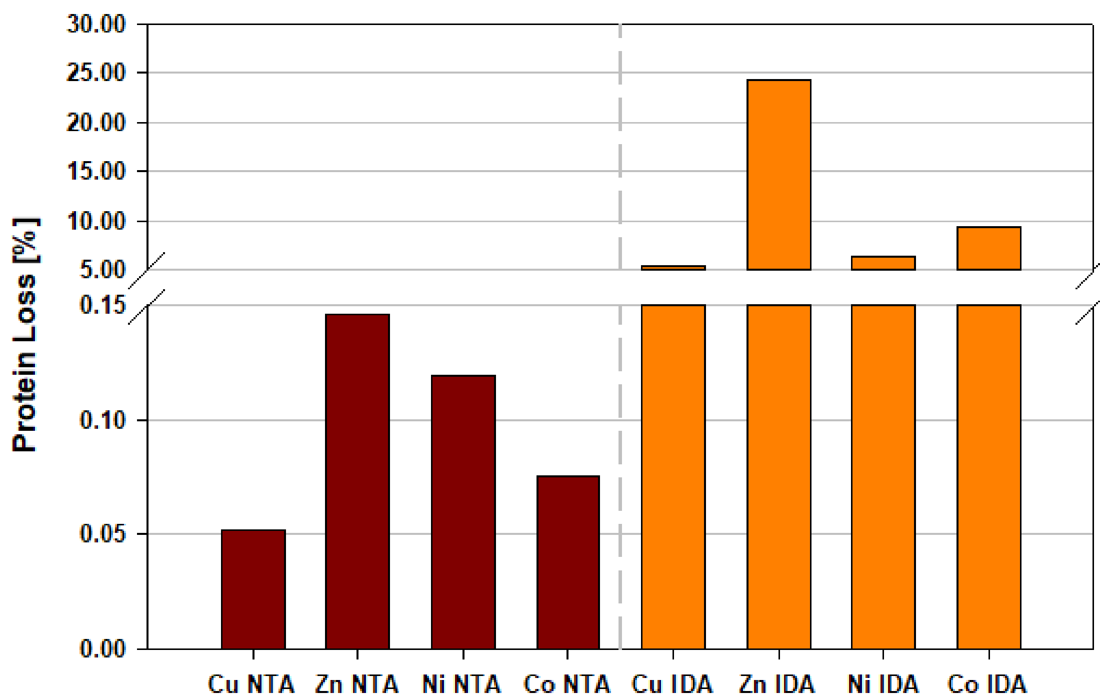

Figure 16: Protein loss during loading and wash on WorkBeads 40 NTA and 40 IDA loaded with different metal ions

Calculating the total recovery, shown in Figure 17, it shows that for NTA coupling, like with IMAC Sepharose, still only around 60-70% could be recovered from the column in all fractions also including flow-through and wash. IDA coupling on the other leads to total recoveries of nearly 100%, meaning hardly any caspase did bind irreversibly to the stationary phase. This may be beneficial at first sight since there is no irreversible binding, but still the caspase is not eluted but lost in flow-through and wash.

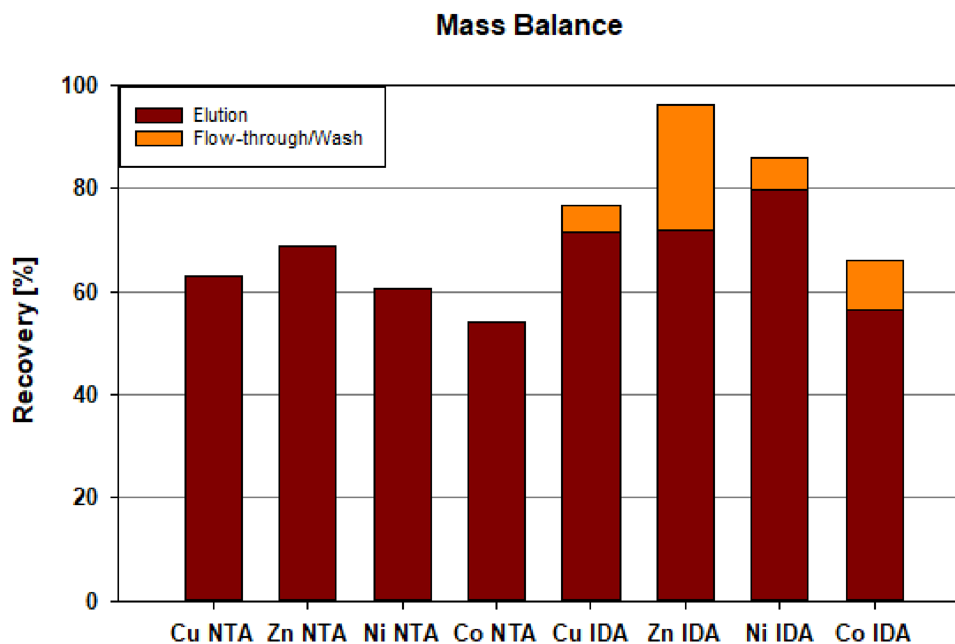

Figure 17: Overview of total recovery in all fractions on WorkBeads 40 NTA and 40 IDA loaded with different metal ions

Comparing the different metal ions tested the performance of cobalt as immobilized metal is quite low with a yield of less than 60% and due to an elevated health and environmental risk is not very attractive for this process. The yield of nickel was a littler lower with the WorkBeads backbone and the NTA ion coupling compared to the results of the loading condition screening, where IMAC Sepharose 6 Fast Flow, also having NTA ion coupling, was used. Coupled via IDA, nickel showed the highest yield of all ions tested in this trial but also a purity of approximately 60%, which could not to be compensated by the higher yield. Zinc performed quite well coupled to NTA as well as to IDA in both yield and purity. Copper had a similar yield to nickel with NTA coupling a slightly lower one with IDA, however the health and environmental risks are lower when using copper. Zinc however shows even fewer risks than copper. This led to the decision to compare zinc to nickel to determine, if the purification behaviour is indeed superior when using zinc as an ion for immobilized metal affinity chromatography.

Figure 18 and Figure 19 show an overview of all samples gained during purification of cpCasp2 using either cobalt or nickel (Figure 18) or copper or zinc (Figure 19).

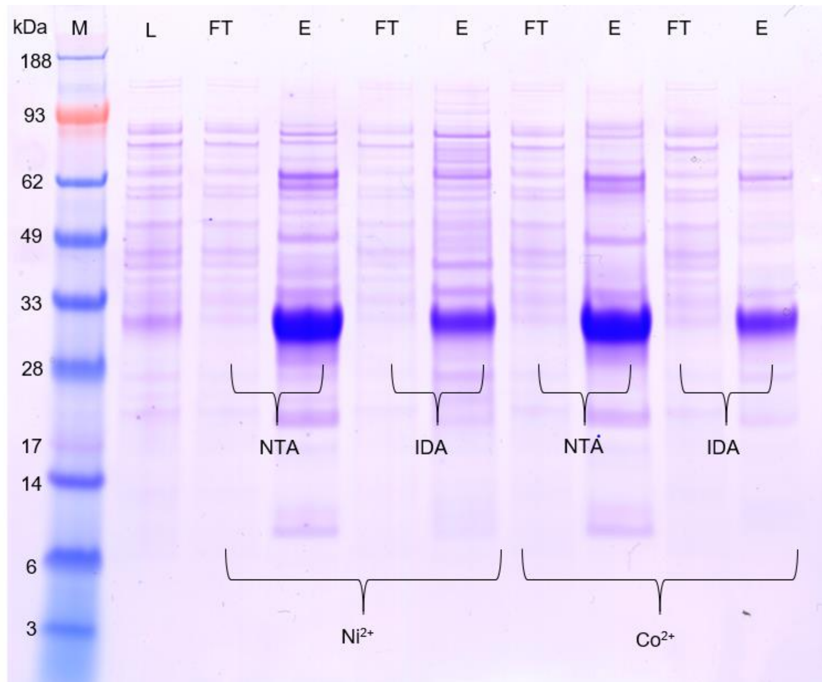

Figure 18: SDS-PAGE of fractions from WorkBeads resin 40 NTA and 40 IDA both with either nickel or cobalt immobilized; M…protein standard SeeBlue Plus2, L… loaded homogenate, FT…flow through, E…elution

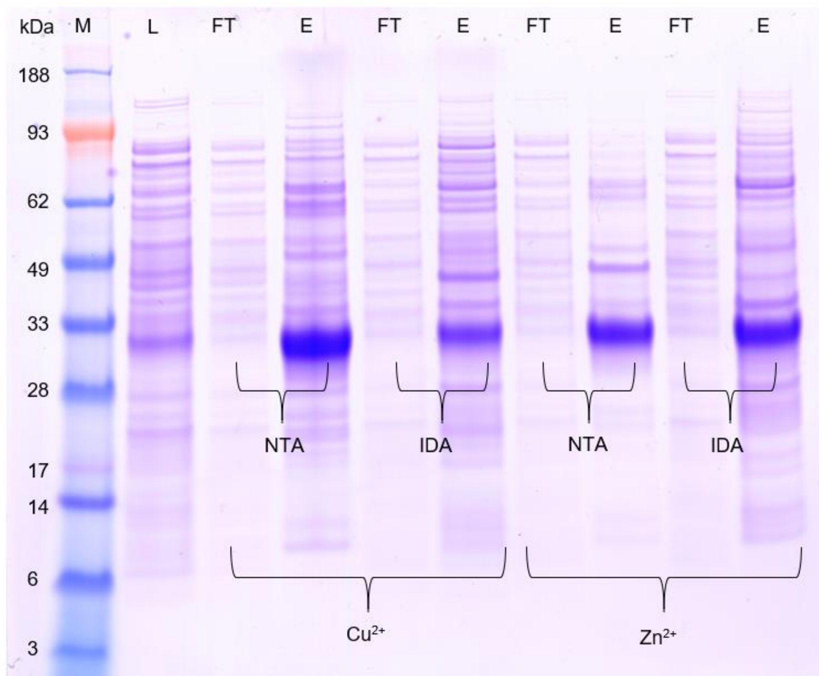

Figure 19: SDS-PAGE of fractions from WorkBeads resin 40 NTA and 40 IDA both with either copper or zinc immobilized; M…protein standard SeeBlue Plus2, L… loaded homogenate, FT…flow through, E…elution

Figure 20 shows the results of zinc immobilized on the WorkBeads resin and the IMAC Sepharose 6 Fast Flow, both having NTA as ligand. Coupled to the latter the yield was approximately 60% whereas zinc coupled to WorkBeads yielded around 70%. The purification runs of both were conducted with 10 mM imidazole during loading, since imidazole has a higher influence on the affinity of his-tagged proteins for zinc than for nickel [35]. Compared to the concentrations used for loading condition screening with IMAC Sepharose, this is rather low. Even 20 mM imidazole during loading was already too high to bind any of the caspase onto the stationary phase. This narrow window led to the conclusion that the sensitivity regarding imidazole concentration is too high to assure a robust process.

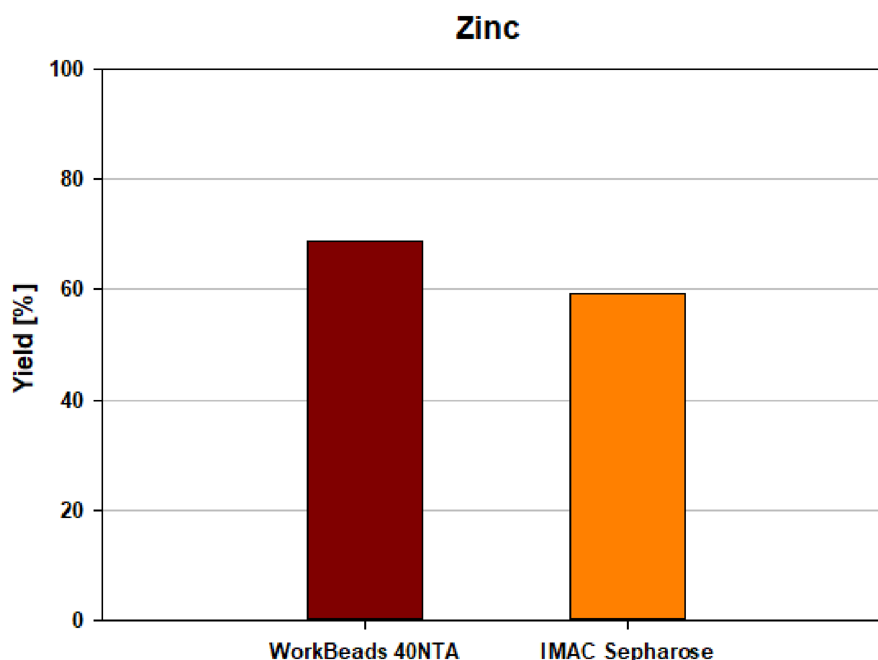

Figure 20: Elution yield of cpCasp2 with zinc immobilized on WorkBeads 40 NTA in comparison with IAMC Sepharose 6 Fast Flow

Immobilization of Nickel to both stationary phases, supported clearly the statement that the WorkBeads resin is pretty sensitive to varying imidazole concentrations, with a drop of yield from 60% with 10 mM to 50% using 30 mM imidazole. No effect can be observed with nickel ions coupled to IMAC Sepharose 6 Fast Flow (see Figure 21).

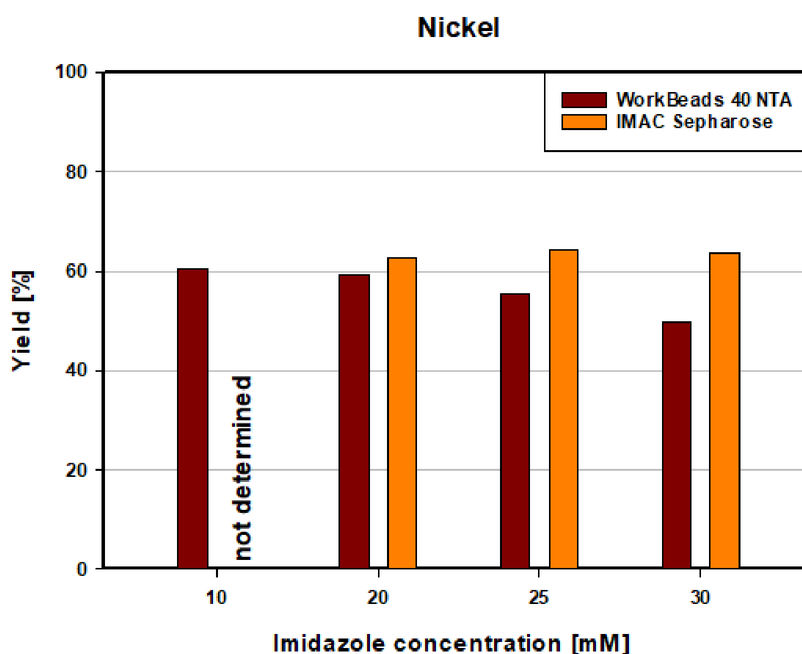

Figure 21: Dependency of yield on imidazole concentration during loading. Nickel ions were immobilized on both stationary phases.

Regarding the stationary phase, coupling mechanism as well as the metal ion immobilized, none of the tested conditions could enhanced the yield while retaining an acceptable purity and being rather insensitive to varying imidazole concentrations during loading making it an improved, robust process. Therefore, Nickel immobilized on IMAC Sepharose Fast Flow coupled via NTA was kept as stationary phase.

4.4.5 Additives

Conformational changes of cpCasp2 during binding to the stationary phase und thus formation of irreversible bonds may be another hypothesis for the rather low yield of the IMAC unit operation.

Therefore, different additives were tested that may influence the conformation of the protein.

Tween 80 reduces the surface tension and additionally shields proteins in solution from attaching to the interphases [36]. Hence it was tested to increase the yield of cpCasp2 by stabilizing the protein and preventing the formation of any irreversible tight bonds. The concentration of Tween used was dictated by the results of the

experiments, which were already performed investigating the optimal concentration of Tween 80 to be added to enhance cleavage performance. With a recovery of 55% (see Figure 22) however, addition of Tween 80 does not show any improvement in step yield.

The reducing agent DTT breaks up di-sulfide bonds in protein structure [37, 38] and yields theoretically in a partial unfolding of the caspase. The concentration to be tested was chosen according to Block et al [14] who reviewed the chemical comparability of different substances with IMAC resins. A maximum of 10 mM of DTT can be used without any effect on efficiency or increased leaching of metal ions [14]. The yield of cpCasp2 using DTT as additive is as good as the reference using no additive (see Figure 22). There is however no improvement and the prevention of altered conformations binding the stationary phase too tightly could not be achieved, given the assumption that this is the reason for the rather low recovery of cpCasp2. Even though yields are quite similar, with DTT having 63% and no additive added having 64%, the decision was made to do not add any of these substances.

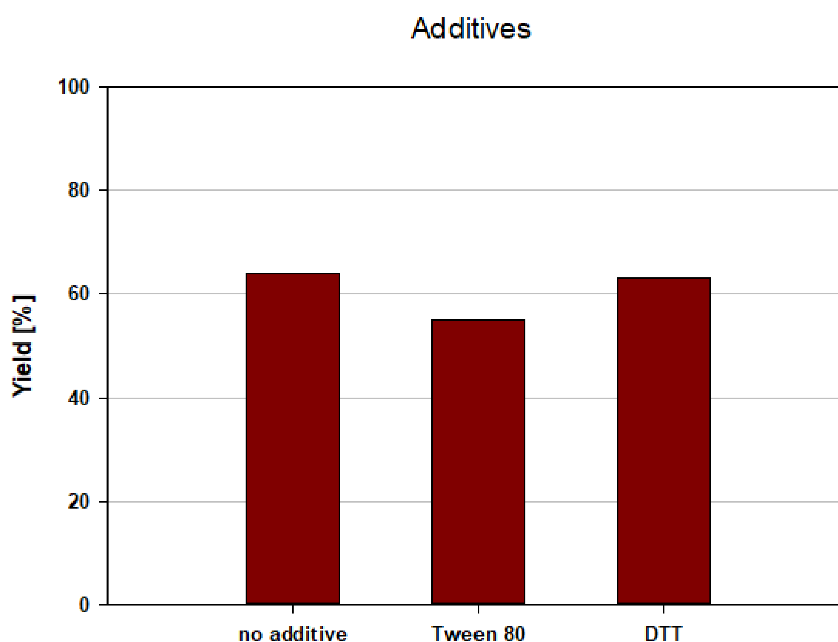

Figure 22: Overview of additives tested: 0.025% Tween 80, 10 mM DTT, and no additive. No additive refers to the optimal conditions investigated in "Screening for loading conditions" only.

Although none of the tested additives did enhance step yield, or purity (purity data not shown) of cpCasp2 purification, there are still numerous other substances that could result in higher yields.

4.4.6 Summary of IMAC optimization

Concluding the optimization for the IMAC capture step using immobilized metal affinity chromatography, it can be stated that only variation in salt concentration as well as pH enhanced the recovery of cpCasp2. Any other approach did not have a superior outcome compared to the results of the loading condition screening. Therefore, by now, the optimized conditions, leading to an increase of step yield from 43% to 63%, are summarized in Table 7.

Table 7: Overview of optimization conditions for IMAC Capture step

Imidazole [mM]	20
NaCl [mM]	300
pH	8.0
metal immobilized	Nickel
Stationary phase	IMAC Sepharose
additives	none

4.5 Mixed mode chromatography

To shorten the process by replacing the cation exchange chromatography with prior buffer exchange with a mixed mode chromatography IMAC capture eluate was loaded directly onto the mixed mode column to see whether cpCasp2 is able to bind to the column. With 40 TREN (Bio-Works, Sweden), it was however not possible to bind any of the loaded protein strong enough. Figure 23 shows clearly that there is hardly any cpCasp2 present in the eluate, but in flowthrough and especially in the wash fraction. This may indicate that the protein somehow interacts with the stationary phase, since it is not leaving the column immediately after loading. Still the retention is not strong enough to keep the protein bound.

Replacing buffer exchange and cation exchange chromatography for polishing with mixed mode chromatography did not work out using WorkBeads 40 TREN. This may have been because salt concentrations were too high or low during loading or

the pH may not have been optimal for this stationary phase. With almost all of the loaded caspase being washed out using the equilibration buffer, maybe another specially designed wash buffer could avoid the unbinding of protein and stationary phase. Mixed mode chromatography works in a complex manner and optimal conditions are crucial to allow binding [26]. In this process though, it is required to load the mixed mode column with the eluate of the capture step without any buffer exchange or any alterations to be made. Otherwise it is not reasonable to replace the already well-established polishing step when the buffer must be exchanged anyways. Finding conditions that may promote protein binding to the stationary phase in this process may be very advantageous since it would lead to a shortened process when replacing two unit operations by a single mixed mode chromatography. Changing of the loading buffer will be of no interest in these optimization approaches but as the results in Figure 20 show, most of the protein is lost during wash. This means cpCasp2 interacts with the stationary phase but not strong enough. Changing the stationary phase or the wash buffer may be first attempts to create a well performing mixed mode chromatographic step in this process.

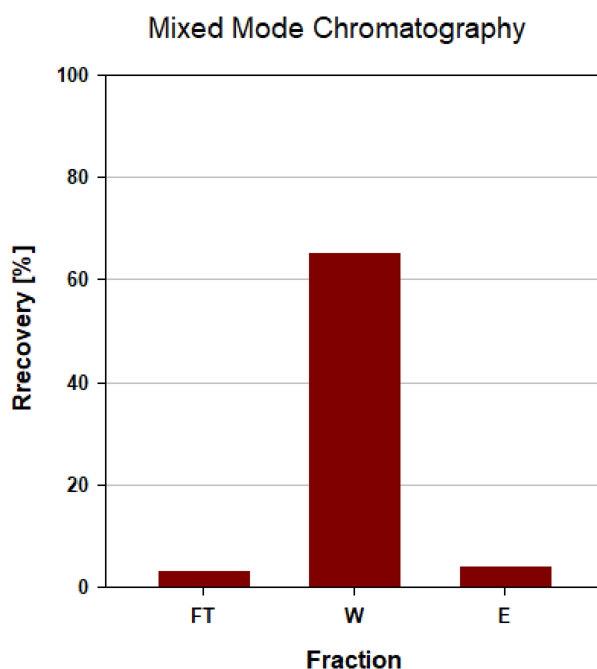

Figure 23: Recovery of cpCasp2 during mixed mode chromatography. FT… flowtrough, W … wash, E … eluate

5 Conclusion

The main objectives of this work were to

(i) find an analytical method to quantify enzymatic activity especially in crude samples

(ii) characterize the initial purification process with determination of step and process yields and identify the unit operations to be optimized

(iii) optimize the chosen unit operations, thus increasing the individual as well as the overall process yield

(iv) shorten the process by substitution of buffer exchange followed by cation exchange chromatography with a single mixed mode chromatographic step

First the FRET-assay used for quantification of the enzymatic activity was adapted to be used as an analytical tool. The approach to reduce potential matrix effects led to the result that addition of EDTA to the assay buffer improved the assay and was hence used for any further analytics. The assay additionally was adapted to function as a routine 96-well-plate assay for fast analytics.

With the functioning assay, the initial process was characterized and the unit operations to be optimized were identified as the cell disruption by high pressure homogenization and the capture step by IMAC. These two unit operations had the lowest step yields with 61% for high pressure homogenization and 43% for IMAC (see Figure 24 – yellow boxes). The overall process yield for the initial process was 17%.

After successful characterization of the initial process, the optimization of high-pressure homogenization and IMAC was performed. Individual process parameters were varied to identify the best performing ones. The green boxes in Figure 24 show the optimized yields. The enzyme recovery of cell disruption by high pressure homogenization could be increased from only 61% to nearly complete recovery of 97%. This was achieved in increasing the operation pressure and keeping the number of passages equal. Regarding protein capture by IMAC the step yield could be increased from 43% to 63% by changing of the buffer composition to a higher pH and a higher salt concentration. By optimizing these two unit operations, the overall

process yield could be more than doubled to 38% instead of 17%. Buffer exchange by diafiltration and cation chromatography were not optimized individually since the step yields of both unit operations was comparably high.

However, there was the approach to substitute buffer exchange and cation exchange by a singly mixed mode chromatographic step. This could lead to and shortened process saving time and resources. The idea was to directly load the eluate from IMAC capture onto the mixed mode column and thus eliminate the need for buffer exchange. In the performed conditions, the enzymes contained in the IMAC eluate could not bind the mixed mode stationary phase strongly enough and were washed out and therefore could not be eluted. This led to the conclusion that with that stationary phase and that wash buffer conditions, it was not possible to further purify cpCasp2 using mixed mode chromatography.

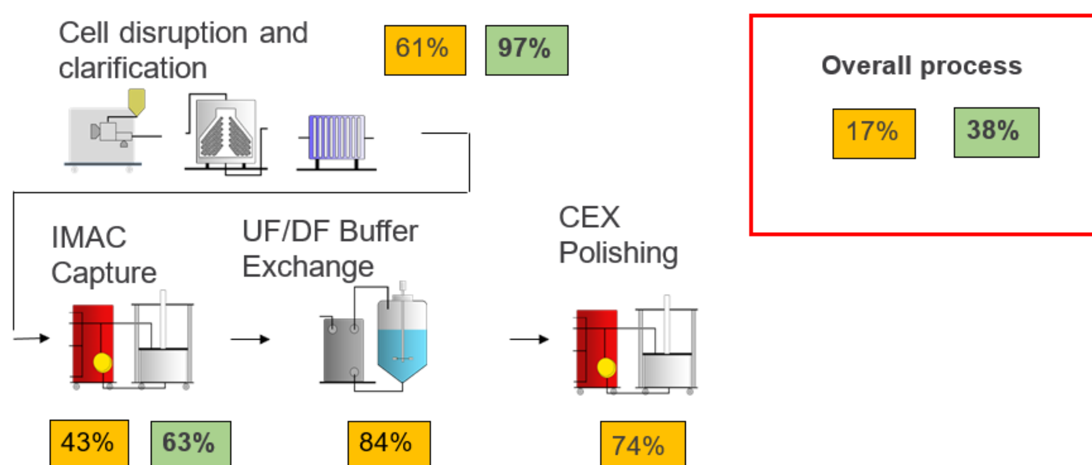

Figure 24: Overview of step and process yield from initial process (yellow boxes) and optimized process (green boxes)

Concluding now the outcomes of this work, 3 of 4 objectives could be fulfilled. An analytical assay to quantify the enzymatic activity was developed and optimized. The initial purification process was characterized, and optimization potential identified. Using this knowledge, the process yield could be more than doubled by improving the performance of high-pressure homogenization and IMAC capture. Shortening of the process by replacing buffer exchange and polishing chromatography by mixed mode chromatography was not possible with the tested conditions.

As an outlook for further work, there is still a lot of potential to make this process even more successful in terms of protein recovery. In IMAC capture there was the main issue of irreversible binding of cpCasp2 to the resin. It now would be very important to identify the mechanism of these strong binding events to find a possibility to prevent it. This could give rise to further improvement of IMAC capture step yields. Regarding mixed mode chromatography different stationary phases and buffers can be tested to find the optimal set of conditions. If the IMAC eluate can be directly loaded onto the mixed mode column and the protein could be retained and eluted afterwards, this new unit operation could replace two others and shorten the process dramatically and also safe resources.

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