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ENZYMATIC HYDROLYSIS OF PHARMACEUTICAL PEPTIDES

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Abstract

Quality control of drugs represents an integral part of the pharmaceutical industry. It verifies the product quality against predefined standards, to guarantee therapeutic efficacy and safety of any medication. For pharmaceutical peptides, the evaluation of the correct chemical composition is essential, because the incorporation of undesired amino acids may lead to unspecified or even harmful metabolic reactions. Quality control of protein-based drugs is currently done by acid hydrolysis. Thereby the peptide is separated into its amino acid subunits and subsequently the composition is analysed via LC/MS. A major drawback of this technique is the racemisation of individual amino acids due to the harsh hydrolysis conditions (6 M HCl, 160 °C). This state-of-the-art methodology will in the future not be able to meet international safety guidelines for pharmaceutical peptides. An entirely new approach is to use proteases, an enzyme family that conducts hydrolytic cleavage, to separate therapeutic peptides into their respective amino acids in a structure-preserving manner.

In the present work the feasibility of enzymatic hydrolysis was investigated by means of the two peptides Lanreotide and Icatibant with the biocatalysts Pronase and Flavourzyme. In order to study enzymatic and acidic peptide hydrolysis quantitatively, photometric methods were established. Reaction parameters of enzymatic hydrolysis were varied, to generate recommendations for an enzymatic hydrolysis protocol. It was demonstrated that a partial enzymatic hydrolysis without racemisation is possible. However, autolysis of the proteases posed a problem, resultant in high amounts of L-amino acids. This in turn biased the determination of the actual hydrolysis efficiency, which was then determined according to artificial and D-amino acids. In comparison, by acid hydrolysis a significantly higher degree of hydrolysis was achieved and the degree of racemisation corresponded to known values from literature. The contribution of autolysis amino acids was reduced by purification of proteases with gel filtration prior to hydrolysis. Still evaluation of the degree of hydrolysis was difficult. In an effort to further reduce autolysis, immobilising the proteases was attempted. Despite an acceptable protein binding, the recovery of enzyme activity on the carrier was marginal. Generated samples of hydrolysates were analysed by LC/MS and the quality of each measurement was assessed. Thereby, batch-to-batch inaccuracies as well as the requirement for a correction of linear trends over time were observed. Based on the low degree of hydrolysis and the strong influence of autolysis, the enzymatic approach is no suitable alternative to acid hydrolysis at the current state of research.

Kurzfassung

Qualitätskontrolle stellt einen zentralen Schwerpunkt in der pharmazeutischen Industrie dar. Sie prüft die Produktqualität gegenüber vorgegebenen Standards, um die therapeutische Wirksamkeit und Sicherheit jeder Medikation zu garantieren. Für pharmazeutische Peptide ist die Evaluierung der korrekten chemischen Zusammensetzung essentiell, da der Einbau unerwünschter Aminosäuren zu unspezifischen oder sogar schädlichen metabolischen Reaktionen führen kann. Qualitätskontrolle von proteinbasierten Arzneimitteln wird derzeit mittels saurer Hydrolyse durchgeführt. Dabei wird das Peptid in seine Aminosäureuntereinheiten zerteilt, und kann anschließend hinsichtlich seiner Zusammensetzung analysiert werden. Ein wesentlicher Nachteil dieser Technik ist die Razemisierung einzelner Aminosäuren während der harschen Bedingungen (6 M HCl, 160 °C). Mit dieser Methodik wird es aber zukünftig nicht möglich sein, internationalen Sicherheitsrichtlinien für pharmazeutische Peptide gerecht zu werden. Ein vollkommen neuer Ansatz besteht nun darin, Enzyme zu verwenden, um therapeutische Peptide auf eine strukturerhaltende Art in die jeweiligen Aminosäuren zu zerteilen. Dafür notwendige hydrolyische Spaltungen können von Proteasen katalysiert werden.

In der vorliegenden Arbeit wird die Machbarkeit enzymatischer Hydrolysen anhand der zwei Peptide Lanreotide und Icatibant und der Biokatalysatoren Pronase und Flavourzym untersucht. Um enzymatische und saure Peptidhydrolysen zu quantifizieren, wurden photometrische Methoden etabliert. Reaktionsparameter der enzymatischen Hydrolyse wurden variiert, um Vorschläge für ein Protokoll enzymatischer Hydrolysen zu generieren. Es wurde gezeigt, dass eine unvollständige Hydrolyse ohne Razemisierung möglich ist. Die Autolyse von Proteasen stellte jedoch ein Problem dar, welches in einer hohen Menge an L-Aminosäuren resultierte. Dies wiederum beeinflusste die Bestimmung der eigentlichen Effizienz der Hydrolyse, welche dann anhand von künstlichen sowie D-Aminosäuren determiniert wurde. Im Vergleich dazu wurde mittels saurer Hydrolyse ein wesentlich höherer Hydrolysegrad erreicht und der Grad der Razemisierung entsprach Werten aus der Literatur. Durch eine Gelfiltrationsreinigung der Proteasen vor der Hydrolyse wurde der aus Autolyse stammende Anteil an Aminosäuren reduziert. Noch immer aber war die Quantifizierung des Hydrolysegrades schwierig. Um die Autolyse weiter zu verringern, wurde versucht die Proteasen zu immobilisieren. Trotz einer akzeptablen Proteinbindung war die Enzymaktivität des Immobilisats gering. Die generierten Proben wurden mittels LC/MS analysiert und die Qualität jeder Messung wurde beurteilt. Dabei wurden einerseits batch-zu-batch Ungenauigkeiten beobachtet und andererseits wurde die Korrektur von linearen Trends über die Zeit als notwendig erachtet. Basierend auf dem geringen Hydrolysegrad und dem starken Einfluss der Autolyse stellt der enzymatische Ansatz zum derzeitigen Stand der Forschung letztendlich keine geeignete Alternative zur sauren Hydrolyse dar.

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List of abbreviations

Miscellaneous

AAA	Amino acid analysis
API	Active pharmaceutical ingredient
BSA	Bovine serum albumin
dH_2O	Distilled water
DTT	Dithiothreitol
EC	Enzyme commission
EMA	European Medicines Agency
ESI	Electrospray ionisation
FDA	United Stated Food and Drug Administration
Fmoc	Fluorenylmethoxycarbonyl
HAE	Heredity angioedema
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
I.B.B.	Institute for Biotechnology and Biochemical Engineering - Graz University of Technology
ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IUPAC	International Union of Applied Chemistry
KH_2PO_4	Potassium phosphate
LC/MS	Liquid chromatography mass spectrometry
MWCO	Molecular weight cut-off
Na_2CO_3	Sodium carbonate
NMR	Nuclear magnetic resonance
OPA	*o*-Phthalaldehyde
PB	Potassium phosphate buffer
RT	Room temperature
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSTR	Somatostatin receptors
Std	Standard
QC	Quality control

Amino Acids

Ala	Alanine
Arg	Arginine
Cys	Cysteine
Gly	Glycine
His	Histidine
Hyp	4-Hydroxy-prolin
Leu	Leucine
Lys	Lysine
Nal	2-Naphthyl-alanine
Oic	Octahydroindole-2-carboxylic acid
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thi	2 -Thienyl -alanine
Thr	Threonine
Tic	1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid
Trp	Tryptophane
Tyr	Tyrosine
Val	Valine

Table of Contents

1 Introduction

1.1 Pharmaceutical peptides

Pharmaceutical peptides are described as amino acid monomer chains, shorter than 40 amino acids. Their mode of action is to bind to cell surface receptors, mimic for example hormones and subsequently activate or inhibit intracellular pathways. Hence, metabolic disbalances as seen for example in cardiovascular diseases, can be restored. Therapeutic peptides possess highly valued pharmaceutical characteristics due to their specificity, safety, tolerability and efficacy in humans, superior to the traditional small drug compounds used [1]. To date, over 7000 naturally occurring peptides were found and they witnessed increasing importance in medical practice since the introduction of insulin in the 1920s [2, 3]. Insulin was followed by oxytocin and vasopressin, which where the next peptide representatives. They were synthetically produced in the 1950s which led to Vincent du Vigneaud winning the Nobel Prize in 1955 [4–6]. Development of systems allowing production of recombinant peptides has driven the production of these therapeutics [3]. Accordingly, the peptide therapeutic market has been estimated in 2018 by $ 25 billion, with tendency to double by the year 2027 [7].

Recombinant synthesis of peptides has driven the production of these therapeutics. However, research activities in this field decreased over time, because small drug compounds and monoclonal antibodies were seen as more promising. In addition, bottlenecks of pharmaceutical peptides were found [8]. Firstly, they are susceptible to degradation by endogenous proteases which cleave peptide bonds (particularly in the gut). This results in a short half-life via oral administration. To circumvent that, injections were necessary, which made peptide drugs even less appealing. Further drawbacks recognised, were of a technical kind, like time-consuming production, and also of a biochemical kind, namely the poor transport properties through biological membranes [3].

Advances in biochemical processes recently renewed interest in the light of peptides. Namely, alternative carrier systems have been introduced to enable oral intake, biotechnological synthesis has accelerated by high-throughput methods and peptide stability was improved by the incorporation of artificial and D-amino acids [9]. As a result of development of bioinformatics, knowledge about structural properties of receptors allowed the computational design of peptides that imitate antibodies and bind to receptors with higher affinity. Lippow et al. demonstrated this, by designing antibodies with a tenfold higher affinity for an epidermal-growth-factor receptor [10]. Overcoming former key hurdles led to the usage of pharmaceutical peptides not only to treat major societal illnesses of our century like cancer. Additionally, they are

used to treat cardiovascular, inflammatory, immunological and muscular diseases [3, 8]. Peptide drugs can be used in such a broad field of diseases because they mostly act on or downstream of G-protein-coupled-receptors. These receptors are found in the majority of metabolic pathways and mediate nearly all kinds of signal cascades, as outlined by Davenport et al. [11].

Beside these applications, research aims to find alternative approaches to combat viral infectious diseases with pharmaceutical peptides. The currently challenging situation with regards to the coronavirus disease 2019 (COVID-19), has driven these scientific efforts towards peptide drugs. [12]. Promising results were found for influenza virus types, chronic hepatisis B, acquired immunodeficiency syndrome (AIDS), and severe acute respiratory syndrome (SARS) caused by coronavirus 2 [13].

Until now, the use of more than 60 pharmaceutical peptides has been authorised by the United States Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Currently, clinical studies include 150 peptides still in active development. In 2020, 4 new peptides have been approved by the FDA. Of these, two are radiopharmaceuticals (containing the isotopes ^{64}Cu and ^{68}Ga) that target critical pathways in cancer cells, and two are antibody drug conjugates [14].

1.2 Quality control of pharmaceutical peptides

Quality control of active pharmaceutic ingredients (API) represents an indispensable cornerstone to ensure safety and efficacy of drugs [15]. The European Pharmacopoeia the United States Pharmacopoeia and the Japanese Pharmacopoeia comprise the three biggest reference books for pharmaceutical quality control, storage, labelling and drug manufacturing. The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) was implemented by these three institutions to combine regulations globally and agree on worldwide guidelines for good manufactural practise [16]. However, strong competition is faced on a national basis due to varying acceptance criteria. What is more, as peptide-based medication is still a novel sector, guidelines often exclude peptides [15]. A detailed description of current guidelines regarding therapeutic peptides will be given later in Section 1.4. The following analysis techniques are currently confirmed and used to define substance quality and degree of impurities in intact therapeutic peptides.

1.2.1 Current analytical platforms

Therapeutic peptides can be analysed qualitatively and quantitatively on three different levels of integrity. Firstly, the complete peptide can be analysed regarding purity and concentration. Secondly, the peptide can be separated into fragments and resulting patterns can be compared. And thirdly, by hydrolysing the peptide, the correct composition can be verified according to amino acids found in the hydrolysate [17]. An overview about the commonly applied methods used for each level of analysis is given in Table 1. They are explained more detailed in the following.

Table 1: Platforms to analyse peptides on three different levels of integrity. Ticks (✓) indicate most commonly applied methods according to literature.

	LC/MS	HPLC	NMR	Spectrophotometry
Complete peptide	✓	✓	✓	✓
Fragments	✓	✓	✓	
Amino acids	✓	✓		✓

To start with, analysis of the complete peptide is currently done by high performance liquid chromatography (HPLC). This is the most widely used method for peptide quality control. With this retention time-based technique, the peptide of interest is compared to a quantitated reference standard. Depending on the peptide and the objective of analytics, suitable detectors have to be chosen. Amongst those, ultraviolet (HPLC-UV) and refractive-index (HPLC-RI) detectors are commonly used. However, the determination of impurities is difficult because these have to be known to identify and quantify them. Yet, with HPLC the degree of impurity as well as the correct concentration of a substance can be verified up to a sub-picomolar level [17, 18]. Similar to HPLC, peptides can be analysed by liquid chromatography coupled to mass spectrometry (LC/MS) allows identification of a substance according to its retention time and its monoisotopic mass. Therefore, analytes are first separated by LC, further ionised by for example electrospray ionisation (ESI) or chemical ionisation and subsequently detected and identified by MS. This technique constitutes the gold standard for metabolite analysis of all kinds and is applied not only for intact peptide analysis but also for fragments and amino acid analysis [18]. Still, limitations arise which are described later on.

Another method to analyse the complete peptide is nuclear magnetic resonance (NMR). NMR determines the distance between atomic nuclei, which allows to overcome the limitation of unknown structural interactions within the peptide [19]. If a conformational modification is present in a therapeutic peptide, it affects effectiveness and safety of the drug. Shortcomings of

NMR are the time-consuming measurement, high costs and extensive data processing. Therefore, NMR is not used as a routine method for quality control, but rather applied for development of new peptides [18, 19]. Since spectrophotometric methods have been established in the 1960s, they have mostly been replaced by chromatographic methods but have been applied to evaluate batch-to-batch consistency during the production process of peptide drugs. Furthermore, moisture content and counterion analysis (e.g. acetate, HCl) are performed on a spectrophotometric basis. These methods often use biuret or ninhydrin as complexation reagents [15, 17].

Peptide fragments are commonly analysed either by NMR or by tandem mass spectrometry. This method is an extension of LC/MS, because after ionisation, two mass spectrometry detectors analyse the samples. The peptide is fragmented directly during the analysis process and resultant fragmentation patterns are then compared. By that, consistency of a target peptide sequence can be verified. A drawback of this method is insufficient reproducibility of mass spectrometric results, which is why peptide fragmentation is not as widespread, compared to complete peptide or amino acid analysis [20].

The third level of peptide quality control is amino acid analysis (AAA), which is mostly done via chromatographic methods like HPLC, or LC/MS. Since each amino acid has a chiral variant, separation of these two forms mostly require derivatisation with ninhydrin and *o*-phthalaldehyde (OPA) serve as such derivatisation agents [15]. Furthermore, stereoselective columns are available which separate zwitterionic molecules like amino acids without derivatisation [21]. Photometric determination of the amino acid content is not a part of routine quality assessment but generally applied in research. It is done by adding complexation reagents like ninhydrin, OPA or 2,4,6 – trinitrobenzenesulfonic acid to the hydrolysate, that react with primary amines in solution. The produced chromophore has an absorbance maximum at a specific wavelength, which is then measured spectrophotometrically [22, 23]. Despite the predominant usage of LC/MS or HPLC in this context, photometric methods indeed have some benefits. Their fast and low-cost procedure gives quick results, which is an advantage to chromatographic methods that face several limitations.

1.2.2 Limitations of LC/MS

Although LC/MS constitutes the analytical high-end tool for molecular component studies, its accuracy and reliability between or within different measurements is still limited [24]. These inaccuracies derive primarily from ion suppression, enhancement of the ESI source, environmental changes of the LC/MS system and sensitivity to salts. Ion suppression and enhancement

of the ESI source are linked to the interactions between analytes of interest and the co-eluting sample matrix, also termed matrix effects [25, 26]. It was found that trivalent salts like phosphate or sulphate, arising from the sample matrix, can cause non-linearity of calibration curves. Subsequently, this affects accuracy of analysis significantly, as reported by Lu et al. [27]. These findings coincide with previous studies from the I.B.B. where non-linearity in LC/MS measurements was observed in combination with inorganic salts [28, 29]. The addition of isotope labelled amino acids as internal standards is a suitable method to detect LC/MS relevant measurement issues. High costs of these ^{13}C labelled metabolites limits the routine usage, though [30].

1.3 Acid hydrolysis – a tool for peptide hydrolysis

Prior to AAA, peptides need to be separated into their amino acid subunits. Stein and Moore first introduced acid hydrolysis as a suitable tool to achieve this separation [31]. For this method, the peptide is incubated at high temperatures for several hours in an acidic solution in an evacuated tube. Usage of 6 M HCl is often recommended, however addition of phenol, *β*-mercaptoethanol [32], propionic acid [33] or hydriodic acid [34] were named as well in literature. Incubation conditions vary, as for example temperatures between 108 °C – 180 °C and incubation times of 15 min – 64 h have been investigated. Short incubation times (15min – 2 h) at high temperatures (> 160 °C) gave the highest degree of hydrolysis (80-100%), which is defined as the proportion of cleaved peptide bonds in a protein hydrolysate [31–36]. In general, the protocol of JOANNEUM RESEARCH HEALTH is closely related to the specified recommendations.

1.3.1 Limitations of acid hydrolysis

A major drawback of acid hydrolysis with which one is concerned is structural and chiral instability of individual amino acids. According to Adebiyi et al. and Inglis et al. this affects mainly L-Trp and L-Cys, resulting in either a total degradation of these amino acids, or conversion into other closely related derivatives [32, 37]. Adebiyi et al. reported, that by adding 3% phenol and 1% *β*-mercaptoethanol to the 6 M HCl solution and conducting acid hydrolysis (of lectine) at 165°C for 4 h, the degree of hydrolysis increased and L-Trp was not destroyed. A degree of hydrolysis of 82 ± 27% was determined without, and 98 ± 16% with the addition of phenol and *β*-mercaptoethanol. In addition, only a third of L-Trp available was destroyed, compared to 100%, prior to addition of phenol. Acidic treatment of free and protein-bound L-Trp showed that recovery was by 5.2% worse in protein bound than in free L-Trp, indicating that the hydrolysis reaction contributed to L-Trp being destroyed [32].

Beside the addition of stabilising chemicals to the hydrolysis solution, alkaline hydrolysis is regarded as an alternative to acid hydrolysis for the determination of L-Trp. As L-Trp is a basic amino acid, it tends to be unstable in an acidic surrounding. By using sodium hydroxide or lithium hydroxide as reagents to hydrolyse protein, L-Trp was stable and could be quantified completely. Alkaline hydrolysis was conducted in 4.2 M alkali at 110°C for 20 h. However, L-Cys was usually destroyed with this approach [38].

Further shortcomings often overseen, are hazards for employees in laboratories. Acid hydrolysis includes a certain risk for burning injuries, inhalation or spillage to skin and eye. Reducing these potential risks necessitates safety precautions, or at best completely avoiding harsh conditions in the analysis process [39].

1.3.2 Racemisation

Another well-known issue of acid hydrolysis is that it induces a change of chirality in amino acids, known as racemisation. Thus, the measured concentration of the enantiomer might either include or lack a fraction that derived from racemisation of the other enantiomer of the same amino acid. To complicate matters, the rate of isomerisation during acid hydrolysis depends on the position of the amino acid in the peptide, sample matrix and molecular structure of the complex [40]. Racemisation follows a reversible first order reaction, therefore conversions of L- to D- as well as D- to L-amino acids can be observed. According to Csapó et al., racemisation events occur 20-80% more often during the acid hydrolysis reaction of protein-bound amino acids, than by acidic treatment of free amino acids [36]. This finding coincides with previously explained instability of protein-bound amino acids during acid hydrolysis. Furthermore, it was seen that change of chirality happens 1.2-1.6x more often with the method first introduced by Moore and Stein in 1963 (6 M HCl, 110 °C, 20 h) [31], than when hydrolysis is conducted within 30 – 45 min at 160 – 180 °C [36]. Degree of racemisation was different for each amino acid investigated but accounted for 1 – 10% according to Kaiser and Brenner (6 M HCl, 110 °C), and 1 – 6% according to Csapó et al. (6 M HCl, 160 – 180 °C) [41, 36].

1.4 Guidelines for pharmaceutical peptide analysis

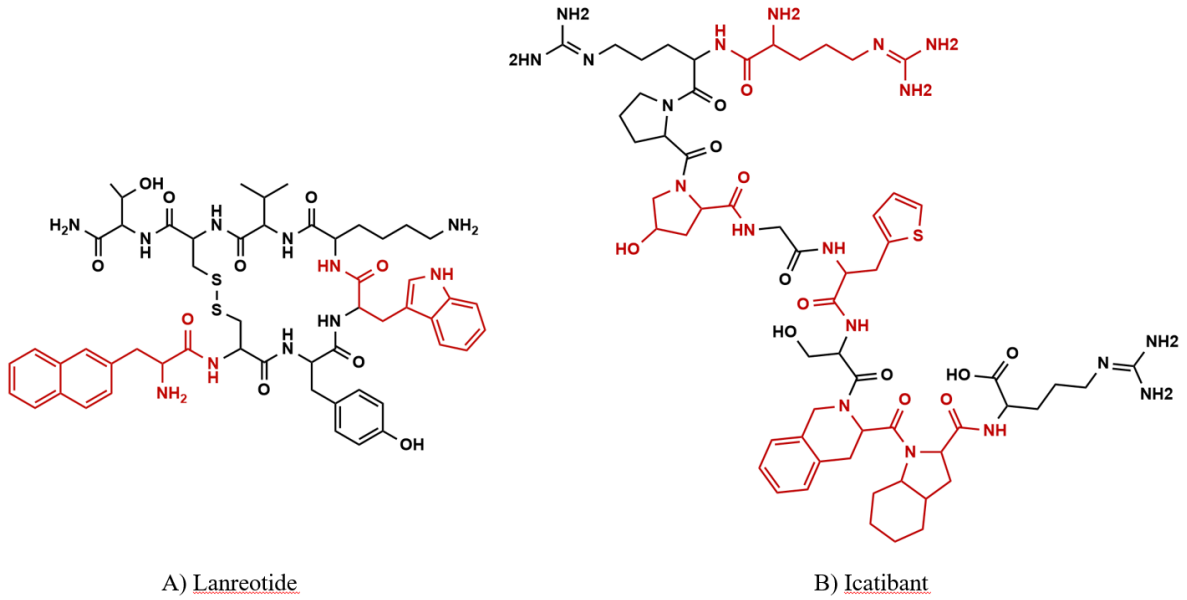
The increasing interest of the pharmaceutical industry in peptide therapeutics is a catalyst for the development of regulations and quality standards in this area. Peptide therapeutics were excluded from general regulations for APIs until the early years of 2000, due to the lack of suitable impurity analysis methods, but they gained ground just in recent years. Therefore, they are now included in standardised regulations and detailed investigations regarding impurities and potential toxicity are expected to increase [42]. Furthermore, it is expected that acceptance

criterions will be broadened and thresholds for impurities will further decrease [15]. Similar to any other medical product, development and approval processes follow four pre-defined steps: 1. Preclinical investigation, 2. Clinical investigation, 3. Post approval marketing surveillance and 4. Life-cycle management [43]. Beside general specifications necessary for APIs like correct labelling, description of the mode of action, information about composition (amino acid primary sequence), manufacturer, stability and safety, details about immunogenicity of peptide APIs either from the peptide or from peptide impurities have to be provided in addition [43]. Quality control as outlined in the following, concerns the third step of the development approval process (3. Post approval market-surveillance).

According to FDA regulations included in the newest ICH guidelines, low level impurities arising in the synthetic process of peptides which are consistent from batch-to-batch generally pose minimal pharmacological or toxicological risks for patients. These impurities are accepted in recently developed drugs, unless the content of impurities exceeded those of a reference listed drug product [43]. The 2021 report of ICH "Impurities in Drug Substances", defines in the so called "Q3A, Q3B and Q6A guidance": If the content of impurities is less than 0.1%, the peptides are suitable for submission. In case of an impurity level up to 0.5% it has to be justified, that the impurity does not affect safety and effectiveness of the peptide intake for the consumer [44–46]. Special regulations are applied to so called orphan drugs that are in use for the treatment of rare diseases (<50 000 patients), as for example Icatibant [43]. Detectable impurities of any kind have to be noted and classified as either identified and quantified, identified, or unidentified [44].

1.5 Peptides of interest

In the present study, the two peptides Lanreotide and Icatibant were used to investigate feasibility of enzymatic hydrolysis. In the following, they are described in detail and their structural formula is depicted in Figure 1.



A) Lanreotide B) Icatibant

Figure 1: Structural formula of Lanreotide and Icatibant. Artificial (D-Nal, L-Hyp, L-Thi, D-Tic, L-Oic) and D-amino acids (D-Trp, D-Arg) are depicted in red

1.5.1 Lanreotide

Lanreotide is applied for the treatment of multiple medical conditions. Acromegaly and gastroenteropancreatic neuroendocrine tumours are the major traits cited in this context [47]. Basically, this might be explained due to the fact that Lanreotide is an analogue of human somatostatin. Somatostatin binds with high affinity to G-protein-coupled somatostatin receptors (SSTR) which regulate, and predominantly inhibit the release of growth hormones from somatotropic cells in the pituitary gland. Thus, in the gastrointestinal tract and in pituitary tumours these receptors are mainly expressed [48, 49]. The malfunction of the regulatory properties of somatostatin, results in an increased production of growth hormones which provokes tumour magnification [50]. Lanreotide is characterised by a longer half-life of about two hours compared to two or three minutes of its competitor somatostatin [48]. What is more, Lanreotide binds with a higher affinity to specific SSTRs which additionally decreases growth hormone release [47].

Regarding structure, Lanreotide is an octapeptide with the following amino acid sequence: H-D-Nal-L-Cys (1)-L-Tyr-D-Trp-L-Lys-L-Val-L-Cys (1)-L-Thr-NH_2. (1) indicates that the two L-Cys build a disulphide bridge. D-Trp and D-Nal constitute non-proteinogenic amino acids, whereas D-Nal is an artificial variant of L-Ala and D-Trp represents the chiral form of L-Trp [51]. The drug was first accepted by the EMA in 2013 and by the FDA in 2007 [52, 53].

1.5.2 Icatibant

Icatibant is used for the successful treatment of heredity angioedema (HAE). Absence or dysfunction of C1-esterase inhibitor is an autosomal dominant trait that results in this rare disorder. It affects 1:10 000 to 1:50 000 people worldwide which makes Icatibant a so called orphan drug, used for the treatment of rare diseases [54]. Generally, three types may be distinguished. Type I is characterised by a deficiency of C1-inhibitor (85% of cases), in Type II C1 inhibitor is dysfunctional (15% of cases) and in the very rare type III the C1-inhibitor is intact (10% of cases) and is linked to an X-chromosomal inhibitor regulation deficiency [54–56].

C1-esterase inhibitor acts as a major inhibitory component of bradykinin production. Disturbance in this pathway causes accumulation of bradykinin, leading to increased vascular permeability, plasma diffusion and oedema formation. This further results in unpredictable angioedema including swelling, pain and redness of the gastrointestinal tract, skin and upper airways. Untreated HAE may cause a life threat, if respiratory areas are affected. [54].

Icatibant is a selective, competitive, proteomimetic bradykinin B2 receptor antagonist consisting of ten amino acids (H-D-Arg-L-Arg-L-Pro-L-Hyp-Gly-L-Thi-L-Ser-D-Tic-L-Oic-L-Arg-OH), whereas five of them D-Arg, L-Hyp, L-Thi, D-Tic and L-Oic constitute non-proteinogenic amino acids. D-Arg represents the chiral variant of L-Arg, and the remaining four amino acids are artificial ones [57]. Due to Icatibants high structural similarity to bradykinin, it blocks binding of bradykinin to its receptor and further mitigates symptoms of oedema. Bradykinin cleaving enzymes do not recognise Icatibant, which promotes endurance of the inhibition [54, 56]. The EMA approved Icatibant with the proprietary name Firazyr® in 2008 for patients suffering from HAE [58] The FDA approved the same product in 2011 [59].

1.6 Proteases – suitable candidates for peptide analysis

Proteases are enzymes that catalyse the cleavage of peptide bonds in proteins in a hydrolytic reaction. According to the enzyme commission (EC) of the International Union of Pure and Applied Chemistry (IUPAC) they are classified as hydrolases (E.C. 3.4.X) [60]. Among the vast group of these proteases (synonymous as proteinases, peptidases) two subclassifications are distinguished. The first classification is based on IUPAC agreements, which divides these enzymes into exoproteases (E.C. 3.4.1 - 3.4.19) and endoproteases (E.C. 3.4.21 - 3.4.24 and 3.4.99). Exopeptidases act either on the N- or the C-terminal end of proteins and are therefore called amino- or carboxypeptidases. The resultant products of this proteolysis, are free amino acids. In contrast to exopeptidases, which cleave from the outside, endopeptidases cleave peptide bonds from the inside of proteins resulting in polypeptides [61]. The second classification according to MEROPS database [62, 63], categorises proteases with regard to their amino acid- and cosubstrate – preferences. Aspartic (A), asparagine (N), cysteine (C), glutamic (G), metallo (M), serine (S), threonine (T), mixed (P) and unknown (U) type peptidases may be distinguished. Amino acid preference often includes a triade (e.g. Asp-His-Ser in serine peptidases), while cosubstrate preference includes e.g. zinc or cobalt which is for example needed in active sites of metallo peptidases [63].

1.6.1 Enzymatic hydrolysis - practical applications of proteases

Proteases account for about 60% of the global enzyme market, which is estimated by $ 1.5 – 1.8 billion per year [64]. Their enormous diversity enables applications in a wide field of industrial and pharmaceutical purposes. Therefore, proteases could also be part of the analytical toolbox in quality control [65].

The industrial protease market accounts for the largest proportion, of which the most dominant use is the detergent industry ($ 1 billion per year) [64]. Since the first commercial use of proteases subtilisin and alkalase from *Bacillus* species in 1963 by Novo Industry, Denmark (Novozymes), the company holds 48% of the total market share [65]. Research for proteases used in the detergents industry tackle challenges like pH stability in alkaline solutions with pH values of 11-12, genetic modifications of alkaline proteases containing more non-oxidisable amino acid moieties (Ser, Ala, Leu) for increased longevity, as well as adaptions for higher catalytic activity in lower temperatures for coLD-water washes at 10 – 20°C [66, 67]. Beside detergents, proteases are widely used in the food industry. In cheese making acidic aspartic proteases are used as milk clotting enzymes with associated release of whey [68]. Proteases are furthermore used in baking to hydrolyse gluten, which accelerates the dough making process and changes

flavour [61]. Protease hydrolysates of soy protein, whey or casein are widely used as food additives, ranging from fortifying fruit juices, cured meat, soups and sauces to increasing digestibility of infant formula and reducing allergenicity of food compounds like peanuts [69]. Beyond these applications, proteases are also used in leather manufacturing, where proteases more and more take the place of conventional chemicals to solubilise hair roots [67]. Based on their high hydrolysis efficiency, proteases are therefore attractive candidates to prevent employees from being exposed to harmful agents. This argument is not only in leather industry but also in this work a major point in order to replace aggressive chemicals [64]. Table 2 summarises studies where amongst others, Flavourzyme and Pronase were used for enzymatic hydrolysis of common proteins, which underlines the potential of these proteases to hydrolyse therapeutic peptides.

Table 2: Proteases used in enzymatic hydrolysis studies. Abbreviations: BSA, bovine serum albumin

Protease	Substrate	Hydrolysis conditions	Degree of hydrolysis [%]	Reference
Pepsin, Pronase	Ovomucin	100° C, 5 min	90	[70]
Flavourzyme, Alcalase	Potato Pulp	85°C, 15 min	44	[71]
Flavourzyme	Whey protein	75°C, 15 min	44	[72]
Pronase, Aminopeptidase	BSA	37°C, 24 h	75	[73]

The broad use of these catalysts in the medical field led to the increase of their production at an industrial scale. They are predominantly used as thrombolytic therapeutics and, in the prevention of heart attacks due to their ability to dissolve clots in blood vessels [74]. Proteases can also be used for the treatment of haemophilia, where more efficient blood clotting is induced due to cleavage of precursor clotting factors [75]. Patients with cystic fibrosis often suffer from severe malfunction of digestion and absorption of nutrients. Nowadays, effective treatment with pancreatic proteases allows near-normal nutrient uptake [76, 77]. The neurotoxin produced by *C. botulinum*, is widely known for its use in cosmetics and contains a metallo protease that hydrolyses neurotransmitter transporters. Hence, it is used to treat spasms and muscle disorders by inhibiting stimulus transfer by the neurotransmitter in the nervous system.[78].

In order to reuse their catalytic properties in a stable manner and fixate these enzymes on specified locations on solid carriers, immobilisation is one of the most successful methods proposed.

For proteases, covalent binding on supports like glutaraldehyde, chitosan or chitin was already confirmed in several studies, to be a suitable immobilisation technique [79–83].

For medical purposes microbial proteases are superior to sources like plant, animal, fungal or bacterial proteases, due to their fast growth and simplicity of handling [66]. In this work two protease extracts, one from a fungal source (Flavourzyme) and one from a bacterial source (Pronase) were used. For the sake of simplicity, we used the term protease for both, Flavourzyme extract and Pronase extract in the present thesis.

1.6.2 Flavourzyme

Flavourzyme is a peptidase blend extracted from *Aspergillus oryzae*. As the name "Flavourzyme" suggests, the major application of this protease is to intensify flavour by modifying food components by hydrolysis [84]. Merz et al. introduced an automated nine-step purification method in 2015, which allowed to characterise 8 enzymes [85]. The identified compounds were 2 aminopeptidases, 2 dipeptidyl peptidases, 3 endopeptidases and 1 α-amylase, of which only the 2 aminopeptidases had a specificity for L-Leu. Molecular size of the proteases in Flavourzyme reached from 25 to 50 kDa. Flavourzyme was most stable and active at 50°C and pH profiles showed maximal activity between pH 7.5 – 9.0, depending on the peptidase, except for α-amylase activity, for which a pH optimum between 5.0 and 5.5 was reported [85].

1.6.3 Pronase

Pronase is sold as a mixture of 10 proteases produced in *Streptomyces griseus*. Proteases present in the blend were first identified in 1968 by Nomoto et al. [86], namely 5 Ser aminopeptidases, 2 endopeptidases, 2 Leu aminopeptidases and 1 carboxypeptidase. Optimal conditions for Pronase activity were pH values between 7.0 – 8.0, and temperatures between 35 – 40 °C [86]. Pronase was already used in a comparative study to investigate the efficiency of hydrolysing bovine serum albumin (BSA) either with 6 M HCl, at 110° C or enzymatically with Pronase for 48 h at 37°C. A hydrolysis degree of 25% and 75% was achieved for Pronase alone and Pronase used together with others in a cocktail of proteases (see Table 2). Yet, complete hydrolysis was achieved by the acidic treatment [73].

1.6.4 Autolysis

Proteolysis is a major tool of living cells to regulate activation or degradation of proteins available. The most common occurrence of proteolysis are post translational N-terminal modifications as for example, removal of signal sequences for transport, proteolytic digestion of e.g.

neurotransmitters by the proteasome and proteolytic activation of precursor enzymes (zymogenes) like the cleavage of proinsolin to the active insulin [87].

Autolysis describes two phenomena: Firstly, autolysis can be a self-digest of a protease, where the enzyme cleaves itself. Secondly, autolysis also describes the circumstance when proteases use other proteases in close proximity as substrates and thus cleave amino acids off their neighboured catalysts. In both cases, these autolytic reactions result in truncated enzymes or the production of free amino acids, which entails a loss of enzyme activity [87]. Chen et al. reported that the autolysis rate increases with a rise of temperature in the tested range (20 – 50 °C) of a protease from *Pseudoaltermonas* [88]. Additionally it was reported, that complete removal of the protease poses a problem, since their autolysis by-products remain in the digested hydrolysate [89]. This characteristic of proteases has to be considered when working with proteases and amino acid analysis in combination, because amino acids could likely derive from the enzyme itself.

1.7 Outline of this Master's Thesis

At present, quality control of therapeutic peptides entails various drawbacks. Acid hydrolysis requires harsh conditions (6 M HCl, 160 °C), which leads to the destruction and racemisation of amino acids, while being hazardous for humans at the same time. In contrast to that, proteases are capable of hydrolysing peptide bonds at moderate temperatures (50 °C) and neutral pH conditions (pH 7.0). In addition, the already versatile regulations for accepted peptide impurities are expected to become even stricter and the pharmaceutical industry lacks innovative methods for quality control of therapeutic peptides. Since little is known about the potential of proteases to hydrolyse pharmaceutical peptides, as an alternative to acid hydrolysis, the following project goals were defined to study its feasibility.

1. Establishment of methods to analyse protease activity and enable monitoring of hydrolysis experiments.

- Improvement of a protease activity assay using casein as substrate.
- Adaption of a photometric assay to determine amino acid content in hydrolysates.

2. Development of a hydrolysis protocol for proteases and study of peptide hydrolysis, catalysed by two different protease mixtures (Flavourzyme, Pronase) under different reaction conditions.

- Preliminary testing of free amino acid stability during acid and enzymatic hydrolysis conditions.
- Investigation of the current acid hydrolysis protocol followed at JOANNEUM RESEARCH HEALTH, to compare the two methods.
- Realisation of enzymatic hydrolysis and analysis of amino acids in samples using LC/MS and the photometric assay.
- Data processing of LC/MS results, including outlier-handling of calibration curves and trend correction.

3. Identification of bottlenecks and development of improvement strategies

- Determination of the most effective purification method for proteases using three common purification devices.
- Choice of suitable carrier and conditions for immobilisation according to protein binding efficiency.
- Analysis of activity measurement with immobilised proteases.

This Master's Thesis was carried out in cooperation with the Institute of Biotechnology and Biochemical Engineering at Graz University of Technology and HEALTH, Institute of Biomedicine and Health Sciences of JOANNEUM RESEARCH Forschungsgesellschaft mBH Graz.

2 Materials

2.1 Instruments

Centrifugation

Centrifuge 5415R Fixed angle rotor F-45-36-8, 36 x 0.5 mL	Eppendorf AG (Hamburg, Germany)

Spectrophotometry

Spectrophotometer DU800 Peltier temperature controller System and applications software version 3.0, Build 5	Beckman Coulter Inc. (Fullerton, USA)
FLUOstar Omega plate reader	BMG Labtech (Ortenberg, Germany)
96-Well Microtiter™ Microplate	Thermo Fisher Scientific (Carlsbad, California, USA)
Semi-micro cuvettes, 1.6 mL, layer thickness 10 mm	SARSTEDT AG & Co. KG (Nümbrecht, Germany)

Pipettes

PeqPETTE, 5 mL	PEQLAB Biotechnologie GmbH (Erlangen, Germany)
Pipette tips, 5 mL	PEQLAB Biotechnologie GmbH (Erlangen, Germany)
PeqPETTE 10 µL, 20 µL, 100 µL, 1000 µL	PEQLAB Biotechnologie GmbH (Erlangen, Germany)
Pipette tips, 10 µL, 200 µL, 1000 µL	Greiner, Bio-One GmbH (Frickenhausen, Germany)
Fisherbrand™ Glass Pasteur Pipette cotton plugged, 270 mm	Thermo Fisher Scientific (Waltham, USA)

Balances

Analytical Balance LE244S 240 g, d=0.1 mg	Sartorius (Göttingen, Germany)
Analytical Balance Entris 224-1S 240 g, d=0,1 mg	Sartorius (Göttingen, Germany)
Balance Acculab VIC-612 Vicon 610g, d= 0.01g	Sartorius (Göttingen, Germany)

LC/MS devices

HPLC Dionex Ultimate 3000 Pump DGP 3600 MIC Flow Manager 3300 MIC 1X2P-10P Autosampler WPS3000 TPL RS	Thermo Fisher Scientific (Waltham, USA)
Heated electrospray ionization source (HESI)	Thermo Fisher Scientific (Waltham, USA)
Mass spectrometer – LTQ Orbitrap XL	Thermo Fisher Scientific, Finnigan (Waltham, USA)
HPLC vials 200 µL, TopSert TPX-Short with glass inlet	Thermo Fisher Scientific (Waltham, USA
Column Astec CHIROBIOTIC T, 5 µm	Astec® Supelco Sigma-Aldrich (St. Louis, USA)
Column Chiralpak ZWIX (-), 3 µm	Astec®Supelco Sigma-Aldrich (St. Louis, USA)

Miscellaneous

Curwood Parafilm „M“ ®	Bemis™ (Neenah, USA)
Digital dual-timer C5080	TFA Dostmann (Wertheim-Reicholzheim, Germany)
Eppendorf reaction tubes 1.5 mL, 2 mL	Eppendorf AG (Hamburg, Germany)
Beaker 25 mL, 500 mL	VWR International (Pennsylvania, USA)
BD Filtration syringe, 10 mL, 0.45 µM MWCO	Becton Dickinson U.K. Itd (Oxford, United Kingdom)
Multivap® Nitrogen Evaporator	Thomas Scientific (New Jersey, USA)
Essential Power Supply mA700	Merck Millipore (Burlington, USA)
Mini Rocket Shaker PMR 30	Grant Instruments Ltd
Thermometer 3001 EKT	Heidolph Instruments GmbH & Co. KG Schwabach, Germany)
Magnetic stirring hotplate MR 3001	Heidolph Instruments GmbH & Co. KG Schwabach, Germany)
Magnetic stirring hotplate with temperature control MR 3001 K	Heidolph Instruments GmbH & Co. KG Schwabach, Germany)
Chemically resistant vacuum pump, MPC 101Z	Ilmvac GmBH (Ilmenau, Germany)
NAP™ - 10 illustra purification column	GE Healthcare (Chicago, USA)

NuPAGE TM 4-12% Bis-Tris Gel 15 wells	Invitrogen Thermo Fisher Scientific (Carlsbad, California, USA)
pH meter 691	Metrohm (Herisau, Switzerland)
Paper weighing boats smartBoat™, size S	Sigma-Aldrich (St. Louis, USA)
Pure-A-Lyzer Dialysis Kit 500 mL, 3.5 kDa MWCO	Sigma-Aldrich (St. Louis, USA)
Circular tube rotator SB3	Stuart equipment (Staffordshire, United Kingdom)
Sarstedt screw cab tube, 50 mL, 15 mL	SARSTEDT AG & Co. KG (Nümbrecht, Germany)
Thermal shaker compact	Eppendorf AG (Hamburg, Germany)
Universal Oven U, 70° C	Memmert GmbH + Co. KG (Schwabach, Germany)
Refrigerator 4° C Essential - 20°C Essential	Philipp Kirsch GmbH (Willstätt-Sand, Germany)
Ultrafiltration tube Vivaspin 500, 5000 MWCO, 10 000 MWCO	Sartorius (Göttingen, Germany)
Vacuum hydrolysis tube, 1 mL	Invitrogen Thermo Fisher Scientific (Carlsbad, California, USA)
Volumetric flask 10 mL, 25 mL, 50 mL	DWK Life Sciences (Wertheim, Germany
Vortex shaker REAX top	Heidolph Instruments GmbH & Co. KG Schwabach, Germany)
Waterbath type 1083	GFL Gesellschaft für Labortechnik GmbH & Co. (Burgwedel, Germany)

Software

Chemdraw v 20.0	Perkin Elmer (Waltham, USA)
CorelDRAW 2021 v 23.0.0.636	Corel Corporation (Ottawa, Kanada)
Microsoft Excel 2016 v 2105	Microsoft Corporation (Albuquerque, USA)
Sigmaplot v 10.0	Systat Software GmbH (Erkrath, Germany)
Xcalibur™ v 4.3	Thermo Fisher Scientific (Waltham, USA)

2.2 Chemicals

Table 3: List of amino acids applied.

Amino acids	Degree of purity	Manufacturer
L-Amino acids		
Ala	≥ 99.0%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Arg	≥ 98.0%	Sigma-Aldrich (St. Louis, USA)
Cys	≥ 98.0%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Gly	≥ 98.5%	Sigma-Aldrich (St. Louis, USA)
Hist	≥ 99.0%	Fluka, Honeywell International Inc. (New Jersey USA)
Leu	≥ 98.0%	Sigma-Aldrich (St. Louis, USA)
Lys	≥ 97.0%	Fluka, Honeywell International Inc. (New Jersey USA)
Met	≥ 99.5%	Fluka, Honeywell International Inc. (New Jersey USA)
Phe	100.2%	Sigma-Aldrich (St. Louis, USA)
Pro	≥ 99.5%	Sigma-Aldrich (St. Louis, USA)
Ser	≥ 99.0%	Fluka, Honeywell International Inc. (New Jersey USA)
Thr	≥ 99.9%	Sigma-Aldrich (St. Louis, USA)
Try	≥ 99.9%	MERCK (Darmstadt. Germany)
Tyr	≥ 99.9%	MERCK (Darmstadt. Germany)
Val	≥ 99.9%	MERCK (Darmstadt. Germany)
D-Amino acids		
Ala	≥ 99.0%	Fluka, Honeywell International Inc. (New Jersey USA)
Arg	≥ 98.0%	Supplied by Joanneum Research HEALTH (Graz, Austria)
Asp	≥ 99.0%	Sigma-Aldrich (St. Louis, USA)
Pro	≥ 99.0%	Sigma-Aldrich (St. Louis, USA)
Ser	≥ 99.0%	Fluka, Honeywell International Inc. (New Jersey USA)
Thr	≥ 99.0%	Fluka, Honeywell International Inc. (New Jersey USA)
Tyr	≥ 98.0%	Supplied by Joanneum Research HEALTH (Graz, Austria)

Table 3: List of amino acids applied. (*continued)*

Amino acids	Degree of purity	Manufacturer
Artificial amino acids		
H-*β*-(2-Thienyl)-L-Ala-OH (L-Nal)	≥ 99.0%	Supplied by Joanneum Research HEALTH (Graz, Austria)
H-*β*-(2-Thienyl)-D-Ala-OH (D-Nal)	≥ 99.0%	Supplied by Joanneum Research HEALTH (Graz, Austria)
L-Octahydroindole-2-carboxylic acid (L-Oic)	≥ 99.0%	Supplied by Joanneum Research HEALTH (Graz, Austria)
Fmoc-L-Octahydroindole-2-carboxylic acid (Fmoc-D-Oic)	≥ 99.0%	Supplied by Joanneum Research HEALTH (Graz, Austria)
L-1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid (L-Tic)	≥ 99.0%	Supplied by Joanneum Research HEALTH (Graz, Austria)
D-1,2,3,4-Tetrahydroisoquinoline-3-carboxylic acid (D-Tic)	≥ 99.0%	Supplied by Joanneum Research HEALTH (Graz, Austria)
trans-4-Hydroxy-L-prolin (L-Hyp)	≥ 99.0%	Supplied by Joanneum Research HEALTH (Graz, Austria)
cis-4-Hydroxy-D-prolin (D-Hyp)	≥ 98.0%	Supplied by Joanneum Research HEALTH (Graz, Austria)

Table 4: List of chemicals applied.

Reagent	Degree of purity	Manufacturer
Acetic Acid	≥ 99%	Honeywell, Riedel-de Haën AG (Seelze, Germany)
Acetonitrile	≥ 99%	Honeywell, Riedel-de Haën AG (Seelze, Germany)
Ammonium formate	≥ 99.0%	Sigma-Aldrich (St. Louis, USA)
Bovine serum albumin fraction V (BSA)	≥ 98%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Calcium acetate	≥ 93.5 - 94.5%	MERCK (Darmstadt. Germany)
Casein	≥ 99%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Citric acid ($C_6H_8O_7$)	≥ 99.5%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Coomassie Brilliant Blue R-250	-	Invitrogen Thermo Fisher Scientific (Carlsbad, California, USA)
Dipotassium hydrogen phosphate (K_2HPO_4)	≥ 99%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Dithiotreitol (DTT)	≥ 99.5%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Ethanol	≥ 96%	Chem Lab NV (Zedelgem, Belgium)

Table 4: List of chemicals applied. ***(continued)***

Reagent	Degree of purity	Manufacturer
Fluorenylmethoxycarbonyl-chloride	≥ 97%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Folin-Ciocalteu-reagent 2 M	-	MERCK (Darmstadt. Germany)
Formic acid	≥ 99%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Hydrochloric acid solution, 12 M (HCl)	≥ 99%	Sigma-Aldrich (St. Louis, USA)
LDS Sample Buffer (4x)	-	Invitrogen Thermo Fisher Scientific (Carlsbad, California, USA)
Methanol	≥ 99%	Honeywell, Riedel-de Haën AG (Seelze, Germany)
N,N-dimethylformamide	≥ 99%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
o-Phthalaldehyde (OPA)	≥ 97%	Sigma-Aldrich (St. Louis, USA)
Potassium dihydrogen phosphate (KH_2PO_4)	≥ 99%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Potassium hydroxide	≥ 99%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Roti®-Quant	5x concentrated	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Sodium acetate	≥ 99%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Sodium bicarbonate	≥ 99.5%	MERCK (Darmstadt. Germany)
Sodium carbonate	≥ 99%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Sodium citrate dihydrate ($C_6H_9Na_3O_9$)	≥ 99%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Sodium dodecyl sulfate	≥ 95%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Sodium hydroxide	≥ 99%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
Trichloroacetic acid	≥ 99.5%	MERCK (Darmstadt. Germany)
Triethylamine	≥ 99%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)
β-Mercaptoethanol	≥ 99%	Carl Roth GmbH + Co. KG (Karlsruhe, Germany)

2.3 Reagents

Buffers

Table 5: List of buffers used.

Buffers[a]	Concentration [mM]	pH value[b]	Weight [g/L]	Components
Sodium bicarbonate	50	10.5	4.34 / 0.76	$NaHCO_3$ / Na_2CO_3
Potassium phosphate (PB)	5.0	7.0	0.81 / 0.44	K_2HPO_4 / KH_2PO_4
		9.0	0.81 / 0.44	K_2HPO_4 / KH_2PO_4
	10	7.0	0.92 / 0.62	K_2HPO_4 / KH_2PO_4
	50	7.0	4.66 / 3.14	K_2HPO_4 / KH_2PO_4
	500	7.0	46.72 / 31.54	K_2HPO_4 / KH_2PO_4
	500	9.0	87.10 / 1.40	K_2HPO_4 / KH_2PO_4
	700	7.0	65.20 / 44.16	K_2HPO_4 / KH_2PO_4
Citrate	1000	5.5	205.32 / 58.00	$C_6H_9Na_3O_9$ / $C_6H_8O_7$

[a] dH_2O was used to solve each component. [b] Used to adjust pH value: Sodium bicarbonate buffer, 1 M NaOH; PB, 1 M KOH; Citrate buffer, 1 M NaOH.

OPA working reagent

50 mg of OPA were solved in 100 mL of 50 mM sodium bicarbonate buffer pH, 1000 µL of 96% *(v/v)* ethanol and 50 µL of *β*-mercaptoethanol. Aliquots of 10 mL were frozen at – 20° C for further usage.

Bradford reagent

Bradford reagent A for assays of 0.1 – 1.0 mg/mL protein

Roti®-Quant was diluted 1:5 with dH_2O as recommended by the manufacturers protocol.

Bradford reagent B for assays of 20 – 100 µg/mL protein

Roti®-Quant was diluted 1:3.75 with dH_2O as recommended by the manufacturers protocol.

Reagents used for the protease activity assay

1. 0.65% *(w/v)* casein suspension, 5.0 mM or 50 mM PB pH 7.0 as diluent, corresponding to experiment

2. 110 mM trichloroacetic acid (TCA) solution prepared in dH_2O

3. Folin-Ciocalteu-reagent diluted 1:4 with dH_2O

4. 500 mM sodium carbonate solution (Na_2CO_3 solution)

5. 10 mM sodium acetate solution pH 7.5 containing 5 mM calcium acetate

6. 1.1 mM L-Tyr standard solution, using 5 mM or 50 mM PB pH 7.0 as solvent. Heating up to 55°C for 30 min was necessary until L-Tyr was completely solved.

7. Protease samples: To be able to identify initial rates properly, typically 3 different protease dilutions prepared with corresponding buffer used in (1.) were prepared. Favourable protein concentration in activity assay was 3 – 300 µg/mL for proteases applied.

Solutions used for SDS-PAGE

Coomassie Brilliant Blue – R250 staining solution
1 g Coomassie Brilliant Blue was dissolved in methanol 50% *(v/v)* and acetic acid 10% *(v/v)* as recommended by the manufacturers protocol.

Coomassie destaining solution
A solution was prepared containing dH_2O, methanol and acetic acid at a ratio of 50:40:10 *(v/v)* as recommended by the manufacturers proto

HPLC mobile phase solvents

Solvent A
Solvent A contained 70% methanol, 30% H_2O and 0.02% formic acid *(v/v)*.

Solvent B
Solvent B contained 49% *(v/v)* methanol, 49% *(v/v)* acetonitrile, 2% H_2O, 25 mM formic acid and 12.5 mM ammonium formate.

Enzymes

Flavourzyme® Protease from *Aspergillus orycae* P6110 - 50 mL ≥ 500 U/mg (solid) LOT: SLCC0480	Sigma-Aldrich (St. Louis, USA)
Pronase® Protease from *Streptomyces griseus* 1 g ≥ 3.5 U/mg (solid) LOT: 40681923	Roche Diagnostics GmbH (Mannheim, Germany)

Carriers

Chitosan	Carl Roth GmbH + Co. KG (Karls-ruhe, Germany)
Eupergit® C	MERCK (Darmstadt. Germany)
Sepabeads EC-EP/S batch number SA 1P 0907/162	Resindion (Binasco, Italy)

Peptides

Icatibant	Fresenius Kabi (Bad Homburg vor der Höhe, Germany)
Lanreotide	Fresenius Kabi (Bad Homburg vor der Höhe, Germany)

3 Methods

3.1 Bradford Assay

Protein concentration was measured following the Bradford method using the commercially available reagent Roti®-Quant. The principle is based on a colorimetric shift of Coomassie Brilliant Blue G – 250 from brown to blue, which results from basic amino acid residues of L-Arg, L-Lys and L-His building a complex with the dye. Consequently, absorbance shifts from 465 nm to 595 nm, which is measured spectrophotometrically [90]. Two different methods, Bradford assay A and B were applied and for both assays, buffer used to dilute samples was used as reference. Experiments were carried out in triplicates.

3.1.1 Bradford assay A

20 µL of sample were transferred to cuvettes, 1000 µL of Bradford reagent A were added, and the cuvette was vortexed briefly. After 10 min of incubation at room temperature (RT), the sample was vortexed briefly before absorbance was measured spectrophotometrically.

3.1.2 Bradford assay B

Three wells of a 96-well microtiter plate were filled with 50 µL of sample before 200 µL of Bradford reagent B were added. The plate was shaking for 1 minute in the plate reader and absorbance was measured after 5 min incubation at RT.

For each method 10 standards with known concentrations of BSA in a range of 0.1 – 1.0 mg/mL (Bradford assay A) and 5 – 200 µg/mL (Bradford assay B) were prepared in dH_2O. For Bradford assay B a set of 10 standards was prepared in 700 mM PB pH 7.0 in addition. Obtained absorbance values were plotted against BSA concentrations and from the linear part of the plot linear regression analysis was carried out to determine slope and intercept values (see Section 4.1). The resultant relationship (see Equation 1) was used to calculate protein concentrations.

$$Abs = \varepsilon_{A,B_{(1),(2)}} * c * d \qquad \textbf{Equation 1}$$

Abs	*Absorbance*	*[-]*
ε_A	*Extinction coefficient A, obtained from standards for Bradford assay A*	$[\frac{1}{\frac{mg}{mL} * cm}]$
$\varepsilon_{B(1),(2)}$	*Extinction coefficients B, obtained from standards for Bradford assay B. (1) Standards prepared in dH_2O, (2), standards prepared in 700 mM PB, pH7*	$[\frac{1}{\frac{\mu g}{mL} * cm}]$
c	*Concentration*	*Bradford assay A:* $[\frac{mg}{mL}]$ *Bradford assay B:* $[\frac{\mu g}{mL}]$
d	*Path length, 1 cm*	*[cm]*

3.2 OPA assay

The principle of this assay is based on a reaction of OPA with NH_2 - residues of amino acids, catalysed by *β*-mercaptoethanol. OPA consists of one benzene ring and two aldehydes attached to adjacent carbons. These aldehydes are forming a ring complex together with NH_2 primary amine residues, which display an absorbance maximum at 340 nm. The final protocol used in this work was the outcome of various modifications of the protocol from the company Interchim (Montluçon, France) [91] .

Interchim recommended preparing a fresh OPA solution every 2 h. The protocol further advised to add 1000 µL of OPA to 100 µL of sample in cuvettes and measure absorbance after 2 min. The recommended calibration range of amino acid concentration was 0.8 – 12 µM. Therefore, α-acetyl-L-Lys standards should be prepared in sodium bicarbonate buffer in the specified range.

In the final protocol, a fresh aliquot of frozen OPA reagent was thawed, briefly vortexed, brought to RT and used within one day. The reagent was continuously protected from light. To reduce the amount of OPA solution used for the final protocol, 50 µL of sample were transferred to cuvettes and 500 µL of OPA working reagent were added afterwards. After briefly vortexing the cuvette, absorbance was measured after exactly 2 min of incubation at RT in duplicates.

Instead of solely using α-acetyl-L-Lys as standard as recommended by Interchim, for the final protocol ten standards of each amino acid present in Lanreotide and Icatibant were prepared in

sodium bicarbonate buffer. The recommended range of amino acid concentration was increased to 0.05 – 2 mM and absorbance was measured as described. The absorbance values obtained were plotted against the known concentration in each amino acid standard. From the linear range of each plot linear regression analysis was carried out to determine slope values, corresponding to the molar extinction coefficient. Linear regression was forced through y = 0 to avoid minor deviations of intercept. Molar extinction coefficients were obtained for each amino acid with primary amines that occurred in the peptides (compare Table 10). These molar extinction coefficients were used to calculate an average ε for each peptide. This averaged ε was used to calculate amino acid concentration of hydrolysis samples, according to Equation 1.

3.3 Determination of enzyme activity

3.3.1 Procedure of activity measurement

A discontinuous protease activity assay was carried out with reference to the protocol of Sigma Aldrich [92]. When proteases break amide bonds of proteins L-Tyr is liberated amongst other amino acids. L-Tyr residues either free or on the surface of the protein build a complex with Folin-Ciocalteu-reagent. This complex is measured photometrically at 660 nm. With standards of known L-Tyr concentrations an extinction coefficient ε can be determined. This coefficient allows calculation of enzyme activity *EA* by quantifying L-Tyr liberated over time by the protease in $\frac{\mu mol}{min}$.

A protocol reported by Sigma Aldrich was used as a basis [92]. All reactions were carried out at 37°C in a 1.5 mL Eppendorf tube. All centrifugation steps were performed at 13 000 rpm, for 5 min, at RT. Both, casein suspension and the three dilutions of enzyme solution were incubated for 5 min, to pre-heat the components to 37°C. Different to the Sigma Aldrich protocol where different amounts of enzyme solution (0.3, 0.5, 1.0 mL) were added to 5 mL casein suspension, 100 µL of enzyme were mixed with 500 µL casein suspension to initiate the reaction. Thereby substrate and phosphate concentration were kept constant. In contrast to the company's protocol, where 30 min of incubation were recommended, the reaction was stopped after 6 min unless indicated otherwise. This was done by transferring 100 µL of the enzyme-casein suspension to 120 µL TCA, followed by a centrifugation step instead of filtration, to separate insolubles from the supernatant. A blank was prepared as reference, as recommended by Sigma Aldrich, by mixing TCA and enzyme first and adding casein afterwards so that the enzyme precipitated completely. After centrifugation, 100 µL of the supernatant were mixed with 500 µL of Na_2CO_3 and 100µL of Folin reagent. It was tested that 20 min of incubation were sufficient

for a complete colour development, thus, recommended incubation of 30 min was reduced to 20 min. Absorbance was measured photometrically after another centrifugation step. Activity measurement of immobilised proteases was carried out with this protocol, with the following adaptions: To 10, 20, 30, 40 and 50 mg of wet protease-bound carrier, 500 µL of preheated casein suspension were added and placed on a thermal shaker immediately. The reaction was stopped after 5, 10, 15 and 20 min. To avoid settlement of carrier, 100 µL of the reacting carrier-casein solution were taken directly from the Eppendorf tube on the thermal shaker and transferred to 120 µL of TCA immediately. 10, 20, 30, 40 and 50 mg carrier instead of immobilisate served as reference. Folin reaction with the supernatant, withdrawn after centrifugation, was then conducted as described above.

Different to the Sigma Aldrich protocol, in which 5 standards of L-Tyr covering a range of 27.5 – 275.0 µM were used for calibration, 12 standards covering a range of 0.01 – 1.0 mM, were prepared in dH_2O, while dH_2O served as reference. To 200 µL of reference and each standard, 500 µL of Na_2CO_3 and 100 µL of Folin reagent were added. After incubation of 20 min and a centrifugation step, the standards were measured photometrically. Absorbance values recorded were plotted against L-Tyr concentration in each standard. From the linear part (0.02 – 1.0 mM L-Tyr) a slope value corresponding to the molar extinction coefficient was calculated by linear regression analysis (ε = 1.1826 mL mM^{-1}).

3.3.2 Calculation of enzyme activity

Equations 2 and 3 depict calculation of volumetric enzyme activity using spectrophotometrically obtained absorbance values. As enzyme activity was measured in duplicates, with three dilutions, an average was calculated from the values. Outliers that did not represent initial rate were not taken into the calculation. One unit (U) was defined as Folin reagent-colour equivalent to 1.0 µmol L-Tyr released from casein per min in $[\frac{\mu mol}{min}]$ at 37°C. Volumetric enzyme activity was defined as [U/mL] and specific activity was specified as [U/mg] protein.

$$EA = \frac{A_{660} * D * F}{\varepsilon_{Tyr} * t}$$ **Equation 2**

EA	*Volumetric enzyme activity*	$[\frac{\mu mol}{min}]$
A_{660nm}	*Absorbance measured at 660 nm*	[1]
D	*Dilution factor of enzyme*	[1]
F	*Dilution factor considering serial dilutions from enzyme-casein reaction and stop reaction. (13.2)*	[1]
ε_{Tyr}	*Molar extinction coefficient of Folin reaction with L-Tyr standards*	$[\frac{1}{mM * cm}]$
t	*Time of reaction*	[min]

$$F = \frac{V_{TCE}}{V_{E1}} * \frac{V_{CE}}{V_E 2}$$ **Equation 3**

V_{TCE}	*Volume of casein-enzyme reaction after stopping with TCA (220 µL)*	[µL]
V_{E1}	*Volume of enzyme in the casein-enzyme reaction (100 µL*	[µL]
V_{CE}	*Total volume in casein-enzyme reaction (600 µL)*	[µL]
V_{E2}	*Volume of casein-enzyme reaction transferred to TCA to stop the reaction (100 µL)*	[µL]

3.4 Protein size determination (SDS – Page)

14 µL of the protein sample (1 mg/mL diluted in dH_2O) were mixed with 5 µL of LDS sample buffer (4x) and 1 µL of DTT. Samples were incubated for 10 min at 95° C. After a centrifugation step at 13 000 rpm for 2 min at RT, 20 µL of a sample were applied on the gel. As reference an unstained page ruler was used. Electrophoresis was carried out at 200 V for 60 min. Staining was carried out for 1 h with Coomassie Brilliant Blue staining solution and destaining was performed for 3 h with destaining solution to wash away excess unbound dye from the gel, as recommended by a protocol by Thermo Fisher [94].

3.5 Enzymatic hydrolysis of peptides

3.5.1 Enzymatic hydrolysis protocols

For each enzymatic hydrolysis reaction enzyme activity and protein content were determined first. 10 mg/mL of Pronase were solved in corresponding buffer, Flavourzyme solution was used as purchased from the flask. 2 mM peptide stocks were prepared in PB prior to usage. Components were preheated to 50°C prior to usage. To either Lanreotide or Icatibant present in a 1.5 mL Eppendorf tube a defined amount of U/mL enzyme was added (10 U in enzymatic hydrolysis experiments, 0.1 U/mL in stability experiments as reference). Volume was filled up

to 1 mL with corresponding buffer. The Eppendorf tube was briefly vortexed and placed in a thermal shaker at 50 °C under constant shaking at 1000 rpm, for 0 – 24 h.

Stopping of the enzymatic hydrolysis reaction was done either by ultrafiltration in Vivaspin tubes (500 µL hydrolysate added to a Vivaspin tube free from glycerol, protocol in Section 3.5.2) or boiling the Eppendorf tube in dH_2O for 15 minutes in a water bath under constant shaking at 40 rpm. After boiling, samples were centrifuged for 5 min at RT at 13 000 rpm, and 100 µL of the supernatant were used for LC/MS measurements.

Samples were transferred to HPLC autsampler vials either undiluted or in dilutions of 1:2, 1:5, 1:20 and 1:40 in dH_2O and stored at 4° C until measurement. Controls applied were enzyme, peptide or an amino acid mix that represented Lanreotide and Icatibant, incubated respectively in buffer. Unless indicated otherwise, each experiment was done in duplicates and each of the duplicates were measured twice (in two dilutions). The three following protocols were carried out and LC/MS measurements of samples acquired in these protocols are further specified as 1st, 2nd and 3rd LC/MS measurement, respectively.

Enzymatic hydrolysis with non-purified enzyme (1st LC/MS measurement)
10 U of non-purified enzyme, and 1.0 mM of either Lanreotide or Icatibant both prepared in 10 mM PB, pH 7.0 were used in this experiment. The reaction was stopped after 0 and 24 h by applying either boiling or ultrafiltration.

Hydrolysis of Lanreotide – time course experiment (2nd LC/MS measurement)
The reaction contained 10 U of NAP purified enzyme and either 1.0 mM or 0.2 mM of Lanreotide. Enzyme and peptide solutions were prepared in 5 mM PB, pH 7.0. Samples were taken at 0, 3, 6 and 24 h and reaction was stopped by ultrafiltration.

Enzymatic treatment of peptide amino acid mix (3rd LC/MS measurement)
For this protocol an amino acid mix (AA-mix) containing 200 µM of each amino acid present in Lanreotide and Icatibant was incubated instead of a peptide with enzymes under otherwise identical conditions. Concentrations of amino acids present more than once in a peptide (L-Cys for Lanreotide and L-Arg for Icatibant) were adjusted accordingly. Stock solutions of amino acids soluble in dH_2O were prepared in 5 mM PB, pH 7.0 at a concentration of 10 mM. Exceptions were L-Nal, L/D-Tyr, L/D-Tic, L-Oic. Preparation of stock solutions for these amino acids is depicted in Table 6 in panel B. To 25 mL of 5 mM PB pH 9.0 present in 50 mL volumetric flask amino acids were added from stock solutions to a final concentration of 200 µM unless mentioned otherwise in Table 6, panel B. pH value was adjusted to 7.0 with 1 M KOH and the

volume was adjusted to 50 mL with PB. This solution representing all amino acids present in each of the peptides was termed AA-mix and was used for either enzymatic or acidic treatment. Preparation of AA-Mix was also tested with amino acid stock solutions prepared as described in Table 6 panel A, combining the solutions in 5 mM PB, pH 7.0. Adjustment of pH to 7.0 could not be accomplished due to high amounts of HCl and acetic acid. Therefore 5 mM PH 9 was used and amino acid stock solutions were prepared as indicated in Table 6 panel B. AA-mix containing 100 µM of each amino acid (except for L-Cys and L-Arg) were incubated with 1 U of NAP purified enzyme for 24 h. Samples were taken after 24 h and enzymes were separated by ultrafiltration

Table 6: Amino acid stock preparation. Panel A depicts preparation of stocks for the 1st and the 2nd LC/MS measurement. Panel B depicts preparation of stocks used for the preparation of AA-mix and standards for the 3rd LC/MS measurement. Abbreviations: MW, molecular weight; c, concentration; aa acetic acid; WS A, working solution for1st and 2nd LC/MS measurement; WS B working solution for the 3rd LC/MS measurement.

	Amino acids	Counts in peptide	MW [g/mol]	c stock [mM][a]	Solvent	c in WS A [µM]	c [mM][a]	c PB [mM][b]	Used for AA Mix	c in AA mix [µM]	c in WS B [µM]
				A			B				
Lanreotide	L-Cys	2	121.16	10	dH_2O	100	10	5	✓	400	200
	D-Cys	0	121.16	10	dH_2O	15	10	5	-	-	200
	L-Lys	1	146.19	10	dH_2O	50	10	5	✓	100	200
	D-Lys	0	146.19	10	dH_2O	15	10	5	-	-	200
	L-Nal	0	215.25	10	80% aa	15	10	500 [d]	-	-	200
	D-Nal	1	215.25	10	80% aa	50	4	500 [d]	✓	100	200
	L-Thr	1	119.12	10	dH_2O	50	10	5	✓	100	200
	D-Thr	0	119.12	10	dH_2O	15	10	5	-	-	200
	L-Trp	0	204.23	10	dH_2O	15	10	5	-	-	200
	D-Trp	1	204.23	10	dH_2O	50	10	5	✓	100	200
	L-Tyr	1	181.19	10	dH_2O [c]	50	2	5	✓	100	200
	D-Tyr	0	181.19	10	80% aa	15	10	5	-	-	200
	L-Val	1	117.15	10	dH2O	50	10	5	✓	100	200
	D-Val	0	117.15	10	dH2O	15	10	5	-	-	200
				A			B				
Icatibant	L-Pro	1	115.13	10	dH_2O	50	10	5	✓	100	200
	D-Pro	0	115.13	10	dH_2O	15	10	5	-	-	200
	L-Hyp	1	131.13	10	dH_2O	50	10	5	✓	100	200
	D-Hyp	0	131.13	10	dH_2O	15	10	5	-	-	200
	L-Oic	1	169.22	10	80% aa	50	5	5	✓	100	200
	Fmoc-D-Oic	0	391.47	10	80% aa	15	10	5	-	-	200
	L-Ser	1	105.09	10	dH_2O	50	10	5	✓	100	200
	D-Ser	0	105.09	10	dH_2O	15	10	5	-	-	200
	L-Thi	1	171.22	10	80% aa	50	10	5	✓	100	200
	D-Thi	0	171.22	10	80% aa	15	10	5	-	-	200
	L-Tic	0	177.2	10	80% aa	15	10	5	-	-	200
	D-Tic	1	177.2	10	80% aa	50	4	500[d]	✓	100	200
	L-Arg	2	174.21	10	dH_2O	100	10	5	✓	400	200
	D-Arg	1	174.21	10	dH_2O	50	10	5	✓	100	200
	Gly	1	75.07	10	dH_2O	50	10	5	✓	100	200

[a] Values refer to final concentration in amino acid stock. [b] Values refer to concentration of PB to solve amino acids in. [c] For 10 mL of a 10 mM stock solution of L-Tyr was first solved in 1 mL 12M HCl by vortexing for 1 min. Then, the solution was filled up to 10 mL with dH_2O. [d] First solved in 300 µL 80% acetic acid, then filled up to volume with 500 mM potassium phosphate buffer pH 9.0.

3.5.2 Purification of proteases

Three different purification methods were tested to reduce the amount of free amino acids present in the enzyme fractions. For all approaches 10 mg/mL Pronase solved in corresponding buffer or Flavourzyme in solution used as purchased, were tested. Each process was monitored by determining total amino acid content, protein content and enzyme activity.

Purification by Vivaspin tubes

Vivaspin tubes with 5 kDa molecular weight cut off (MWCO) are with a 500 µL inlet that contain a membrane that filters particles in sizes less than 5 kDa. To remove glycerin from an unused Vivaspin membrane, the membrane was washed with 500 µL of dH_2O 3 times by centrifugation at 13 000 rpm for 15 min at RT. 500 µL of enzyme solution (or 500µL hydrolysate, see Section 2.5.1) were transferred to the tube, following repeated centrifugation for 15 min at 13 000 rpm until 300 µL filtrate were collected.

For purification of enzymes, volume of the remaining solution in the tube, filled up to 500 µL with either dH_2O or buffer, followed by another centrifugation step. This purification procedure was repeated three times. To ensure that enzymes did not permeate through the filter, enzyme activity was measured in the supernatant, and as no activity was detected, Vivaspin tubes were verified as tight.

Purification by dialysis

Pur-A-Lyzer dialysis tubes (3.5 kDa MWCO) purify proteins and amino acids due to the principle of diffusion of solutes. The tubes were equilibrated with 500 µL of either dH_2O or 5 mM PB. Dialysis was carried out as described in the manufacturer protocol [95]. Briefly, the dialysis tube was placed in 2 L of either dH_2O or 5 mM PB. Dialysis was carried out for 2 h while gently stirring at 5 rpm in a cooled room at 4° C. In case of an increase of volume in the dialysis tube the volume was documented.

Purification by NAP columns

NAP columns are pre-packed with Sephadex^{TM} and are used for desalting, rebuffering or purifying biological compounds. They require solely gravity to enable the gel filtration process. NAP-5 (0.5 mL sample volume) and NAP-10 (2.5 mL sample volume) were applied with either 5 mM PB or 10 mM PB pH 7.0. Protocols were carried out as recommended by the manufacturer [96].

3.6 Acid hydrolysis of peptides

The protocol was carried out in accordance with a standard operating procedure at JOANNEUM RESEARCH HEALTH. A schematic illustration of the procedure is depicted in Figure 2.

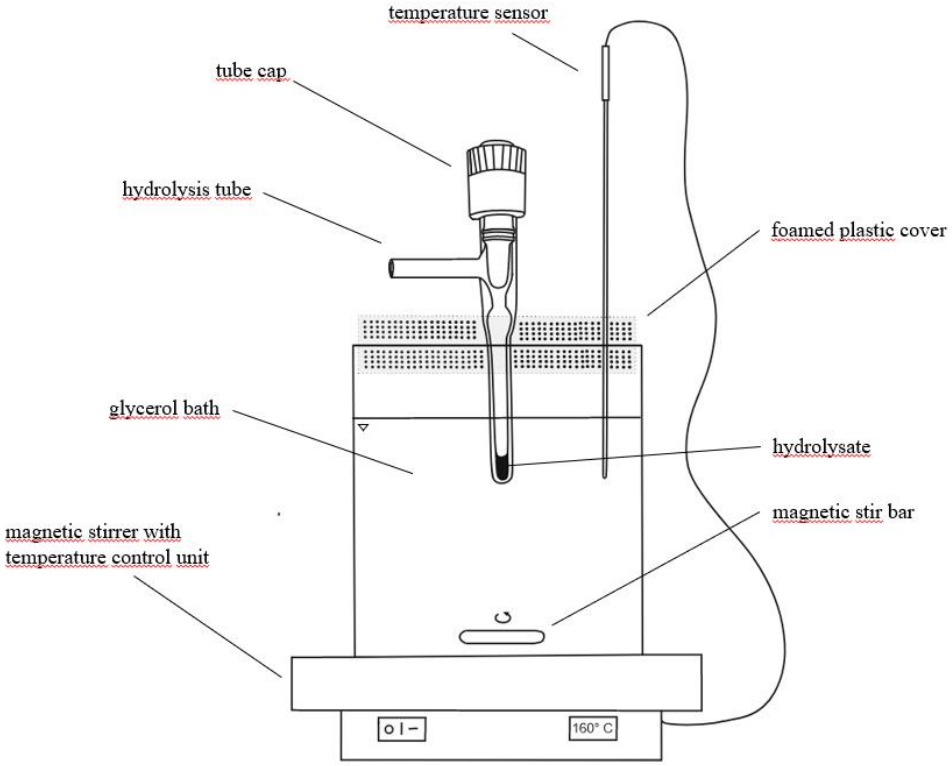


Figure 2: Schematic illustration of acid hydrolysis. Hydrolysis tube fixated with a cover made of foamed plastic (square with dotted pattern), closed with tube cap, tip with hydrolysate (black area) is plunged into the glycerol bath. Temperature control of the magnetic stirrer ensures 160° C hydrolysis temperature, glycerol bath placed on a magnetic stirrer.

A glycerol bath was prepared in a 500 mL beaker and pretempered to 160° C. It was placed on a magnetic heating stirrer connected to a temperature sensor. A solution of 10 mg/mL peptide was prepared in dH_2O from which 100 µL were transferred to a hydrolysis tube. Next, 100µL of 12 M HCl were added to the solution, resultant in 6 M HCl in the final solution. The cap of the tube was first not completely closed. Then the tube was vacuumised with a vacuum pump until no bubbles were observed in the hydrolysis solution before closing the cap completely. The tube was then placed in the glycerol bath as shown in Figure 2 for 30 min. Experiments were done in triplicates.

The reaction was stopped by transferring the tube to a 25 mL beaker containing dH_2O at RT for cooldown for 10 min. Afterwards, the solution was transferred into an empty 25 mL beaker with a glass pipet. The tube was rinsed once with 100 µL 6 M HCl and briefly vortexed, before

adding the residual hydrolysate to the 25 mL beaker. The sample in the beaker was dried completely with a nitrogen evaporator, before 20 mL dH_2O were added. The beaker was placed in an ultrasonic bath for 15 min in order to completely solve the hydrolysate. 100 µL of the hydrolysate (diluted 1:1 and 1:2) were then transferred to an autosampler vial which was stored at 4° C until measurement. Samples obtained from this protocol were measured in the course of the 2nd measurement.

For time course experiments hydrolysis tubes were incubated in the glycerol bath for 10, 20, 30, 40, 50, 60, 75 and 90 min. Experiments were carried out with Lanreotide and Icatibant and samples were measured in the course of the 3rd measurement. No replicates were measured.

3.7 LC/MS measurement

3.7.1 Preparation of standards

The following setup of standard preparation was applied for the 1st and 2nd LC/MS measurement. Amino acid standards were prepared at JOANNEUM RESEARCH HEALTH, according to an internal standard operating procedure "IN HTH METH081". The standards were made to analyse hydrolysis of Lanreotide and Icatibant. They contained all amino acids present in the peptide and additionally all chiral L- or D- variants for each amino acid. First, 10 mM stock solutions of these amino acids were prepared in 10 mL volumetric flasks. Therefore, amino acids were weighed and solved as depicted in Table 4, panel A.

As D-Oic was complexed with Fmoc (Fmoc-D-Oic), a deprotection procedure was necessary prior to preparation of standards. Fmoc-D-Oic was solved in a Triethylamine : N,N -dimethylformamide mixture (ratio 1:1 (vol)) and incubated at RT for 24 h to separate the Fmoc-group from the amino acid. The solution was then vortexed briefly and an aliquot of 400 µL was dried with nitrogen evaporation to complete dryness. The dried D-Oic was taken up in the same volume of 80% (v/v) acetic acid. A deprotection efficiency of 100% was assumed based on a previous report [97]. Note, the Fmoc-deprotection method was not validated by JOANNEUM RESEARCH HEALTH.

Next a working solution was prepared in dH_2O, containing each native (meaning originally present once in the peptide) and each chiral amino acid variant in concentrations specified in Table 6, panel A. This working solution was then diluted according to Table 7 to prepare six standard solutions. In addition, three solutions were prepared from the working solution that contained amino acids in concentrations in between but close to the lower, the middle and the higher standard solutions. These were termed quality control (QC) solutions.

Calibration curves of lower final concentrations for chiral amino acid variants were chosen as 1-10% degree of racemisation during acidic treatment of L-amino acids were reported according to Kaiser and Brenner [41]. In the last step 100 µL of each standard and QC were transferred to LC/MS autosampler vials, labelled properly and stored at 4°C until measurement.

Table 7: Preparation of standards and quality controls applied for 1st and 2nd LC/MS measurement. Abbreviations: c, concentration; WS, working solution; Std, standards; QC, quality control.

Std and QCs	#	WS [µL]	dH_2O [µL]	c native amino acids [µM] [a]	c chiral variant amino acids [µM] [a]
Std	1	1000	0	50.0	15.0
Std	2	840	160	42.0	12.6
Std	3	680	320	34.0	10.2
Std	4	520	480	26.0	7.8
Std	5	360	640	18.0	5.4
Std	6	200	800	10.0	3.0
QC	1	950	50	47.5	14.3
QC	2	500	500	25.0	7.5
QC	3	240	760	12.0	3.6

[a] values refer to final concentration in the Std or QC.

For the 3rd LC/MS measurement L- and D- amino acid standards were prepared separately to ensure that no amino acid contained its chiral variant already in the original containers. Seven calibration points instead of six were prepared, covering a range of 5 – 200 µM for each L- or D-amino acid, by diluting working solution B in 5 mM PB pH 7.0 (see Table 6, panel B). There was only one QC prepared which was done by pooling 500 µL of standard 4 of L-amino acids with 500 µL of the standard 4 of D-amino acids. This QC is further specified as LD-QC. Deprotection of Fmoc-D-Oic was done as described above with the adaption that the nitrogen dried sample was taken up in 5 mM PB.

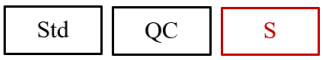
3.7.2 LC/MS measurement of samples

LC/MS measurement was carried out at JOANNEUM RESEARCH HEALTH with a HPLC-MS system from Thermo Fisher Scientific™. All samples were stored on ice during the 20 min transportation from TU Graz to JOANNEUM RESEARCH HEALTH. Prior to measurement autosampler vials were vortexed briefly and if necessary, air bubbles were removed by knocking slightly against the vials. Scheme 1 depicts a general injection sequence for LC/MS measurements done at JOANNEUM RESEARCH HEALTH. Each measurement started with the measurement of standards and QCs. Before and after the standard and the QC set dH_2O was injected. dH_2O was also injected after QCs and after every 10 samples measured. Standards and QCs were measured from low to high concentrations (Std 6-1, QC 3-1) and all samples

were measured in a randomised order. Unless indicated otherwise, the LC/MS sample protocol was completed by injecting standards and QCs in the end, to detect a loss of signal. Two methods A and B were applied. Relevant information is listed in Table 8. All amino acids of Lanreotide could be analysed by Method A while for Icatibant Method B had to be applied together with Method A to cover all respective amino acids.

1st LC/MS measurement:

Sample measurement was performed as shown in Scheme 1. Standards and QCs were solely measured in the beginning. The "µL-pickup" method was used as injection mode.



Scheme 1: Injection sequence of LC/MS for the 1st LC/MS measurement. dH_2O was injected before and after each depicted box and after every 10 samples measured. Abbreviations: Std, standards; QC, quality control; S, sample.

Adaptions for 2nd LC/MS measurement:

Standards and QCs were not only measured in the beginning and in the end but also randomised between the samples after each 4-7 sample injections (see Scheme 2). Additionally, 4 standards each containing the same amount of amino acids (as in Std 3, Table 7) were prepared to receive final potassium phosphate concentrations of 0, 1.0, 2.0, and 5.0 mM. The measurement sequence is depicted in Scheme 2. The "partial loop" method was used as injection mode.

Scheme 2: Injection sequence of LC/MS for the 2nd LC/MS measurement. Abbreviations; Std, standards; QC; quality control; S, sample.

Adaptions for 3rd LC/MS measurement:

The LD-QC was measured seven times throughout the whole measurement after every 10 samples measured. The "partial loop" method was used as injection mode. Scheme 3 depicts the applied measurement sequence.

Scheme 3: Injection sequence of LC/MS for 3rd measurement. Abbreviations: L/D-Std; L/D-standards; LD-QC, L/D-quality control pooled from standard 4; S, sample.

Table 8: LC/MS measurement parameters and approximate retention times of amino acids. Abbreviations: AA, amino acids. NA, not applied.

HPLC parameter	Method A	Method B
Columns used	Astec CHIROBIOTIC T, 5 µm	Chiralpak ZWIX (-), 3 µm
Mobile phase	Solvent A	Solvent B
Gradient	Isocratic	Isocratic
Column Temperature	25° C	25° C
Flow rate	200 µL/min	1000 µL/min
Run time	approximately 17 min	approximately 17 min
Autosampler parameter		
Buffer tubing size	50 µL	50 µL
Syringe size	25 µL	25 µL
Sample volume	2 µL	10 µL
Loop volume	> 10 µL	> 10 µL
Autosampler temperature	5° C	5° C
Wash solvent	100% MeOH	100% MeOH
Injection mode	partial loop[a] or µL-pickup[b]	partial loop[a] or µL-pickup[b]
Icatibant AA	*Retention times [min][c]*	*Retention times [min][c]*
L-Arg	10.0	NA
D-Arg	13.7	NA
L-Pro	NA	7.2
D-Pro	NA	4.9
L-Hyp	NA	6.4
D-Hyp	NA	7.9
L-Ser	4.7	NA
D-Ser	5.2	NA
L-Tic	7.7	NA
D-Tic	12.4	NA
L-Thi	5.3	NA
D-Thi	6.1	NA
L-Oic	NA	13.0
D-Oic	NA	4.7
Gly	5.5	NA
Lanreotide AA		
L-Thr	4.3	NA
D-Thr	4.8	NA
L-Tyr	4.7	NA
D-Tyr	5.4	NA
L-Val	4.4	NA
D-Val	5.5	NA
L-Trp	6.3	NA
D-Trp	5.0	NA
L-Nal	5.4	NA
D-Nal	6.7	NA
L-Lys	8.8	NA
D-Lys	13.5	NA
L-Cys	4.9	NA
D-Cys	6.6	NA

[a] Used for 1st and 2nd LC/MS measurement; [b]Used for 3rd LC/MS measurement.

[b] Constant retention times may vary slightly depending on the actual condition of the HPLC system used (column age, runtime, etc.). Therefore, these values represent approximate values.

3.8 Covalent immobilisation of proteases

3.8.1 Methods of immobilisation studies

The principle of covalent immobilisation is the binding of enzymes to polymeric supports due to functional groups on the catalyst and the carrier. Epoxide groups constitute the binding property of Sepabeads EC/EP-S and Eupergit C. Immobilisation with Chitosan is promoted due to its reactive amino and hydroxyl groups on the surface of the carrier [83, 98, 99] The following protocol was tested in four studies (A, B, C, D) and enabled the determination of a suitable carrier, binding efficiency and specific activity of proteases immobilised on the carrier. Table 9 summarises the parameters examined in the four studies.

Table 9: Immobilisation studies accomplished. Abbreviations: IB, immobilisation buffer; WB, wash buffer; S, supernatant fraction; W, wash fraction; PC, protein content; EA, enzyme activity; c, concentration;

Study	Carrier (200 mg)	Carrier [mg]	c enzyme [µg][a]	NAP[b]	IB [mM]	WB [mM]	Fractions collected	Output[c]
A	Sepabeads EC-EP/S	100	100	no	700	50	S, W1, W2	PC
	Eupergit C		100	no	700	50	S, W1, W2	PC
	Chitosan		100	no	50	50	S, W1, W2	PC
B	Sepabeads EC-EP/S	200	100	yes	700 50	50	S, W1, W2, W3, W4	PC EA[b]
C	Sepabeads EC-EP/S	200	100/200/ 400/800/ 1500	yes	700	50	S, W1, W2, W3, W4	PC EA
D	-	-	100	yes	700	-	-	PC EA

[a] Values refer to total amounts in 2 mL reaction volume. [b] NAP purification was either carried out or not. [c] Output refers to output parameters applied. [d] Varying amounts of beads applied in the activity assay were tested (10-40 mg).

For all studies protein content of proteases used was determined prior to immobilisation (A-D) and after Studies B, C and D. For all studies carrier was transferred to a 2 mL Eppendorf tube in duplicates and two different buffers 50 mM and 700 mM PB were used for the immobilisation (see Table 9). 1 mL of buffer was added to suspend the carrier. 100 – 1500 µg of enzyme either purified with NAP-10 columns or non-purified were added to the suspension. The tube was filled up to 2 mL with corresponding buffer and placed on a circular tube rotator at 40 rpm for 0 – 24h at RT. Carrier and supernatant were then separated by transferring the suspension to a filtration syringe (0.45 µM MWCO) and pushing the liquid through the filter into a weighed Eppendorf tube. This solution was termed filtrate fraction. To wash loosely or unbound enzyme

off the carrier, 1 mL of 50 mM PB, pH 7.0 was added to the remaining wet carrier. Then, the liquid was again pushed through the filter into another weighed Eppendorf tube. This step was repeated 2 – 4 times, to collect wash fractions 1 – 4. Eppendorf tubes were weighed to determine the volume of each fraction and kept on ice for further analysis.

3.8.2 Determining moisture content of Sepabeads EC-EP/S and Eupergit C

200 mg of each carrier were weighed in duplicates in Eppendorf tubes. 1 mL of 700 mM PB was added. Carrier was separated from buffer with a filtration syringe as described in the paragraph above. Two portions of 10 – 20 mg of wet carrier were weighed and placed on pre-dried paper weighing boats. They were placed in a drying oven at 70°C for 24 h until a constant weight was reached. After the drying process the paper boats were weighed again. The difference of wet minus dry carrier weight represented the amount of dH_2O in the carrier. The ratio ($r_{w/d}$) of weighed wet to weighed dry carrier both determined in mg was used for the calculation of immobilisate activity per mg dry carrier. For precise calculation see Section 3.9.2.

3.8.3 Calculation of protein concentration in immobilisation

Calculation of protein bound to the carrier was calculated according to Equation 4. Molar extinction coefficient ε of Bradford assay B obtained from standards either prepared in dH_2O ($\varepsilon_{B(1)}$) or in 700 mM PB ($\varepsilon_{B(1)}$), was applied for all fractions except for supernatant fractions in 700 mM PB. For those fractions, molar extinction coefficient ε ($\varepsilon_{B(2)}$) obtained from standards prepared 700 mM PB was used for calculation of protein concentration in samples. Protein concentration was multiplied by $\Delta w_{B\text{-}A}$. This volume was determined by subtracting the weight of the Eppendorf tube before from the weight after collecting each fraction. Protein contents in each supernatant (*Sup*) and wash fraction (w_x) were summed up for each timepoint, respectively and subtracted from the amount of enzyme initially offered to the carrier. Binding efficiency was calculated in percentage points, assuming total enzyme concentration initially offered to the carrier as 100%.

$$PC_f = PC_i - \sum\left(\frac{\bar{x}_{Abs595}}{\varepsilon_{B(1),(2)}} * \Delta w_{B-A}\right)_{Sup} + \left(\frac{\bar{x}_{Abs595}}{\varepsilon_{B(1),(2)}} * \Delta w_{B-A}\right)_{w_x} \qquad \textbf{Equation 4}$$

PC_f	*Amount of enzyme immobilised on carrier*	$[\mu g]$
$\Delta w_{B\text{-}A}$	*Weight of Eppendorf tube after minus before collecting fraction*	$[mL]$
PC_i	*Enzyme amount offered to carrier*	$[\mu g]$
$\bar{x}_{A595nm}$	*Average of absorbances measured at 595nm*	[1]
$\varepsilon_{B(1),\,(2)}$	*(1) Extinction coefficient of standards prepared in dH_2O* *(2) Extinction coefficient of standards prepared in 700 mM PB*	$\left[\frac{1}{\frac{\mu g}{mL} * cm}\right]$
Sup	*Refers to supernatant fraction*	
w_x	*Refers to wash fractions 1-4*	

3.8.4 Calculation of enzyme activity in immobilisation studies

To account for additional dH_2O deriving from wet carrier, factor *F* was used to adjust water content in the activity assay as described in Equation 5 (F_i). Equation 6 describes the conversion of weighed wet carrier to dry carrier with ratio $r_{w/d}$ determined in Section 2.9.1. Total volumetric activity (EA_i) of the immobilisate was calculated according to Equation 7. Specific activity of immobilisate was determined by [EA_i/µg] protein bound on the carrier.

$$F_i = \frac{V_{TCE}}{V_{E1}} * \left((\Delta w_{B-A}) + V_C\right) \quad \textbf{Equation 5}$$

$$w_d = \frac{W_w}{r_{w/d}} \quad \textbf{Equation 6}$$

$$EA_i = \frac{(\frac{F_i}{\varepsilon_{Tyr} * t}) * \bar{x}_{A660nm} * D}{w_d} * w_i \quad \textbf{Equation 7}$$

F_i	*Dilution factor of serial dilutions from enzyme-casein reaction, stop reaction, and additional dH_2O from beads*	*[mL]*
V_{TCE}	*Volume of casein-enzyme reaction after stopping with TCA (0.220 mL)*	*[mL]*
V_{E1}	*Volume of casein-enzyme reaction transferred to TCA solution to stop the reaction*	*[mL]*
V_C	*Volume of casein suspension added to carrier to start the reaction*	*[mL]*
Δw_{B-A}	*dH_2O in beads: difference of Eppendorf tube weight before minus after collecting fraction (for the solution a density of $1\frac{g}{cm3}$ was assumed to convert the measured weight to mL)*	*[mL]*
$r_{w/d}$	*Ratio of wet carrier to dry carrier*	*[1]*
EA_i	*Total volumetric enzyme activity in immobilisation sample*	$[\frac{\mu mol}{min}]$
w_i	*Weight of dry carrier used for immobilisation*	*[g]*
w_d	*Weight of dry carrier used in activity assay*	*[g]*
ε_{Tyr}	*Molar extinction coefficient of Folin reaction with L-Tyr standards*	$[\frac{1}{mM * cm}]$
$\bar{x}_{A660nm}$	*Average of absorbances measured at 660nm*	*[1]*
t	*Time of reaction*	*[min]*
D	*Dilution factor of enzyme*	*[1]*

3.9 Data Processing

Data analysis was performed in Microsoft Excel 2016, peak integration was done at JOANNEUM RESEARCH HEALTH with the software Xcalibur v 4.3. Graphs shown in this Master's Thesis were established with SigmaPlot v 10.0, chemical structures were made with ChemDraw v. 20.0, schemes and illustrations were drawn with CorelDRAW graphics suite 2021 v.23.0.0.363. From this plot outliers that did not fit the linear relationship were excluded manually, before linear regression analysis was applied. Resultant regression parameters were used to transform all compound-specific signals into molar units

3.9.1 Peak integration

Peaks were identified by Xcalibur according to the monoisotopic mass of each amino acid and their typical retention times (see Table 8). Elution profiles of identified compounds were integrated by Xcalibur software modes Genesis, Avalon or ICIS. If necessary, choice of integration mode and peak border was adjusted manually for each peak. A plot of linear relationship between standard concentration and integrated area was obtained. From this plot outliers that did not fit the linear relationship were excluded manually before linear regression analysis was applied. Resultant regression parameters were used to transform all compound-specific signals into molar units.

3.9.2 LC/MS data processing

After export of obtained concentrations to an Excel file, amino acid standards and QCs were first analysed. If standards and QCs were injected multiple times, values obtained were investigated regarding stability over time. A linear trend correction was done to balance out a variation of signal over time as described below. If this correction showed an improvement of values of standards, it was applied to all samples. Phosphate correction was conducted to account for different phosphate concentrations in dilutions applied.

Linear trend correction

Known concentration of each standard was plotted against measured concentration. Slope ε and intercept d were calculated with the LINEST Excel function. Calculation of corrected values is shown in Equation 8 as X_{corr}. The LINEST function was applied to the complete data set of standards primarily with X_k and X_m and after the correction with X_{corr} and X_k. Correlation coefficient R^2, k and standard error of k were used to further determine improvement of accuracy of the standard concentration signal after linear trend correction. In the last step this correction was applied as indicated to all samples measured.

$$X_{corr} = \frac{(X_m)}{(t*\varepsilon+d)}$$ **Equation 8**

ε	*Molar extinction coefficient*	$[\frac{\mu M}{h}]$
d	*Intercept*	$[\mu M]$
t	*Timepoint of measurement*	$[h]$
x_m	*Measured value*	$[\mu M]$
X_k	*Known value*	$[\mu M]$
x_{corr}	*Corrected value*	$[\mu M]$

Potassium phosphate correction

Concentration values of the four potassium phosphate samples of standard 3 (2nd measurement) were used. Average and standard deviation were calculated from the injections of each molarity class of each amino acid. According to Equation 9 a ratio *r* was calculated from the Std 3 that contained no potassium phosphate ($\bar{x}_0$)and a Std 3 that contained 1.0, 2.5 or 5.0 mM potassium phosphate ($\bar{x}_{1-5\ mM}$). This was done for each amino acid respectively. An error on this ratio (*ER*) was calculated according to Equation 10. Additionally, an upper and a lower bound for *r* were defined by Equation 11. Whether a correction with *r* was necessary for an amino acid or not, was determined by the following criterion: If the upper bound (*ub*) was higher and the lower bound (*lb*) was lower than 1, no correction was carried out for the specific amino acid. Unless this criterion is met, each linear trend corrected sample value was multiplied by *r*.

$$r = \frac{\bar{x}_0}{\bar{x}_{1-5}}$$ **Equation 9**

r	*ratio of averages*	*[-]*
$\bar{x}_0$	*average value of standard 3, containing 0 mM potassium phosphate*	$[\mu M]$
$\bar{x}_{1-5\ mM}$	*Average value of standard 3, containing 1.0/2.5/5 mM potassium phosphate*	$[\mu M]$

$$ER = r * \sqrt{(\frac{s_0}{\bar{x}_0})^2 + (\frac{s_{1-5}}{\bar{x}_{1-5}})^2}$$ **Equation 10**

r	*ratio of averages*	*[-]*
$\bar{x}_0$	*average of standard 3 injections, 0 mM potassium phosphate*	$[\mu M]$
$\bar{x}_{1-5}$	*average of standard 3 injections, 1.0/2.5/5 mM potassium phosphate*	$[\mu M]$
s	*standard deviation*	$[\mu M]$
ER	*error of ratio*	*[-]*

$$ub = r + 2 * ER$$
$$lb = r - 2 * ER$$

Equation 11

ub	*upper bound of average ratio*	*[-]*
lb	*lower bound of average ratio*	*[-]*
r	*ratio*	*[-]*
ER	*error of ratio*	*[-]*

After all corrections were accomplished, concentration values of samples were multiplied with the respective dilution factor. The correct concentration was determined by calculating the average of all injections – depending on the experiment – and the corresponding standard deviation.

4 Results

4.1 Bradford assays

Bradford assay A constitutes the basic method to determine protein concentrations between 0.1 and 1.0 mg/mL. Extinction coefficient obtained from standard absorbances measured was ε = 0.73222 mL mg^{-1}, linear range of the plot was 0.1 – 1.0 mg/mL. Bradford assay B was carried out, because for immobilisation experiments a smaller range (5 – 200 µg/mL) of protein concentrations was needed. Preliminary studies showed different absorbance values for samples prepared in 700 mM PB pH 7.0, than those prepare in dH_2O. Therefore, two different calibrations were applied. Figure 3 indicates absorbance values obtained from standards prepared in either dH_2O ($\varepsilon_{(1)}$ = 0.00553 mL µg^{-1}) or buffer ($\varepsilon_{(2)}$ = 0.01001 mL µg^{-1}). Linear concentration range for Bradford assay B was 5 – 200 µg/mL in dH_2O and 5 – 80 µg/mL in 700 mM PB, pH 7.0.

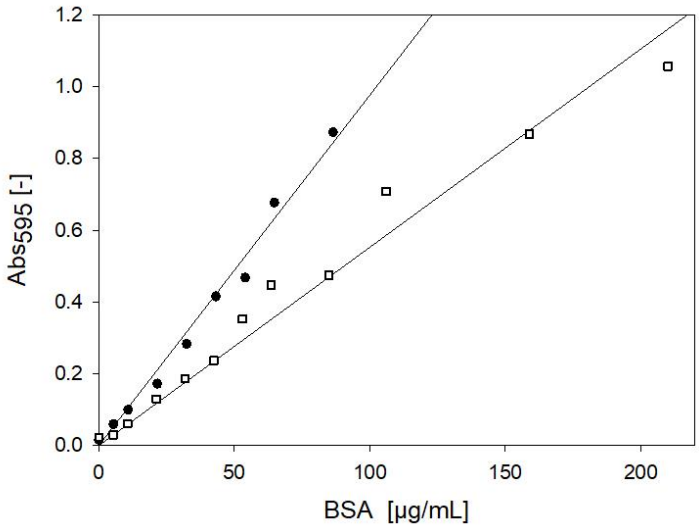


Figure 3: Measurement of standards for Bradford assay B. Each symbol refers to the average of triplicates measured for each BSA standard. Standards prepared in dH_2O depicted as black filled dots, standards prepared in 700 mM PB depicted as empty squares.

4.2 OPA assay

Absorbance values obtained were plotted against known concentrations. Linear regression was applied to the linear part, from which correlation coefficient R^2 and a slope that corresponds to the molar extinction coefficient ε [mL mM^{-1}] was determined. Results are summarised in Table 10. Molar extinction coefficients of all amino acids present in Lanreotide could successfully be determined. For amino acids present in Icatibant, absorbance values were only obtained for L-

Arg, D-Arg, L-Ser and Gly. As expected, OPA reagent did not react with L-Pro, L-Hyp, L-Thi, D-Tic and L-Oic, because instead of a primary amine, these amino acids contain a secondary amine. Correlation coefficients of $R^2 = 0.99$ were determined for all amino acid calibrations except for D-Nal ($R^2 = 0.94$) The average of all molar extinction coefficients for Lanreotide was $\varepsilon = 0.48 \pm 0.09$ mL mM^{-1} (L-Cys was excluded from the calculation due to the strong deviation) and $\varepsilon = 0.48 \pm 0.26$ mL mM^{-1} for Icatibant. These values were used to estimate amino acid concentrations in hydrolysates.

Table 10: Extinction coefficients of amino acids in Lanreotide and Icatibant. Linear range refers to range of concentration from which absorbance values were used to apply linear regression. Abbreviations: AA, amino acids; ε, extinction coefficient; R^2 correlation coefficient, Lan, Lanreotide; Ica, Icatibant.

Lan AA	ε [mL mM^{-1}]	R^2	Linear range [mM]	Ica AA	ε [mL mM^{-1}]	R^2	Linear range [mM]
L-Thr	0.58	0.999	0.05 - 1.99	L-Pro	-	-	-
L-Val	0.50	0.994	0.05 - 2.04	L-Hyp	-	-	-
D-Trp	0.48	0.999	0.05 - 1.98	L-Oic	-	-	-
L-Lys	0.39	0.999	0.04 - 1.75	L-Ser	0.50	0.995	0.05 - 1.99
L-Tyr	0.36	0.999	0.04 - 1.79	L-Thi	-	-	-
D-Nal	0.58	0.946	0.05 - 2.07	D-Tic	-	-	-
L-Cys	0.27	0.994	0.05 - 2.01	L-Arg	0.49	0.999	0.05 - 2.04
				D-Arg	0.48	0.999	0.05 - 1.99
				Gly	0.49	0.999	0.05 - 1.96

4.3 Quality of LC/MS measurements

LC/MS measurement values obtained after data processing with Xcalibur, (see Section 3.9.1) were analysed. It was investigated for which amino acid, measured with either Method A or B, calibration was successful. Further, it was analysed whether time dependent linear trends or effects of KH_2PO_4 were found in standards and QCs. If necessary, corrections were made as described in Section 3.9.2, and improvement of data was analysed in regard of parameters like R^2, linear range, k, and standard error of k (Err_k). With these statements, data acquired for samples could be corrected and interpreted in a better way.

4.3.1 1st LC/MS measurement

Prior to the Xcalibur data processing step it was noticed that elution profiles of amino acids measured with Method B showed peaks that appeared fractured without clear borders. Furthermore, peak signals did not reflect a linear relationship between the standard concentration and peak signal. Therefore, peak integration and calibration of L/D-Pro, L/D-Hyp and L/D-Oic could not be accomplished. L-Cys, L-Ser and D-Arg did not show any relationship between calculated

and known concentration in standards, so linear regression was not applied and calibration could not be accomplished. Performance of Method A was acceptable and data obtained, was analysed after Xcalibur data processing, regarding quality of calibrations. In Table 11, correlation coefficients R^2 with the corresponding linear range are shown for each amino acid, measured with either Method A or B. A linear relationship between LC/MS signal and amino acid concentration in standards was observed in 13 out of 14 amino acids of Lanreotide. For Icatibant this applied to 7 out of 15 standards. All amino acids for which calibration was successful showed a R^2 higher than 0.98, exceptions were D-Tyr (R^2 = 0.928) and D-Cys (R^2 = 0.815). The linear range of all calibratable amino acids covered all standards from the lowest to the highest calibration point. QC samples were only measured in the beginning and corresponded to the known concentration within the 15% of accepted deviation. However, since QC samples were not measured in the end of the measurement, a comparison with the end-quality of the measurement could not be made.

Table 11: Quality of amino acid calibration of the 1st LC/MS measurement. Linear range refers to range of concentration from which absorbance values were used to apply linear regression. Abbreviations: AA, amino acid; R^2 correlation coefficient.

Lanreotide AA	LC/MS Method	R^2	Linear range [µM]	Icatibant AA	LC/MS Method	R^2	Linear range [µM]
L-Thr	A	0.998	10 - 48	L-Pro	B	-	-
D-Thr	A	0.988	3 - 17	D-Pro	B	-	-
L-Val	A	0.987	10 - 50	L-Hyp	B	-	-
D-Val	A	0.992	3 - 15	D-Hyp	B	-	-
L-Trp	A	0.992	3 - 15	L-Oic	B	-	-
D-Trp	A	0.995	10 - 49	D-Oic	B	-	-
L-Lys	A	1.000	10 - 48	L-Ser	A	-	-
D-Lys	A	1.000	3 - 15	D-Ser	A	0.994	2 - 13
L-Tyr	A	1.000	10 - 48	L-Thi	A	0.992	10 - 49
D-Tyr	A	0.928	3 - 15	D-Thi	A	0.995	3 -15
L-Nal	A	0.997	3 - 15	L-Tic	A	0.991	9 - 50
D-Nal	A	0.998	10 - 49	D-Tic	A	0.993	3 - 15
L-Cys	A	-	-	L-Arg	A	0.998	10 - 50
D-Cys	A	0.815	3 - 15	D-Arg	A	-	-
				Gly	A	0.994	10 - 50

4.3.2 2nd LC/MS measurement

In this measurement, each standard and QC sample was measured three times. In the beginning, then after every 4-7 sample injections and in the end, which allowed a quality assessment throughout the whole measurement. After Xcalibur data processing, quality of obtained data was analysed. In this measurement all amino acids could be calibrated and quality of calibration was similar to the 1st measurement but on average correlation coefficient R^2 was lower by 7% . A list containing more detailed information about calibrations of the 2nd measurement is provided in the appendix, Table 20. After calibration and acquirement of data, the quality of the measurement was assessed stepwise. At first, resultant values for QC 1, 2 and 3 were analysed. Results of QCs were all similar and are exemplarily represented by QC2 in Figure 4 which was measured after 2, 16 and 32 h.

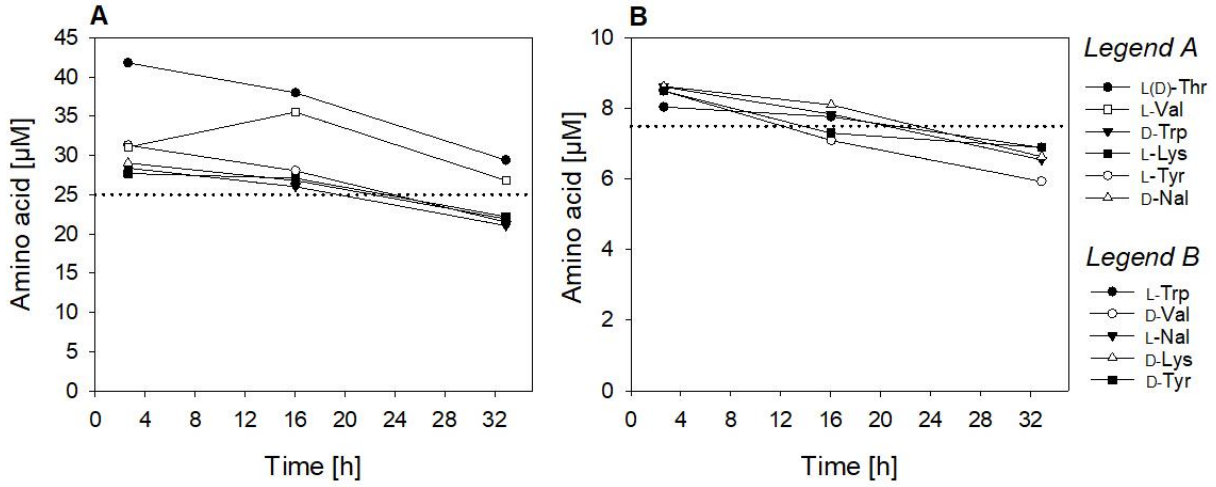


Figure 4: QC2 measured over time. Timepoints: 2 h, 16 h, 32 h. Panel A: Amino acids naturally present in Lanreotide all added at a concentration of 25 µM. Panel B: Possible chiral amino acid variants of Lanreotide, added at a concentration of 7 µM. L-Cys was excluded for a clearer representation of data.

Panel A indicates that those amino acids that were added in the higher concentration (25 µM, L(D)-Thr, D-Trp, L-Lys, L-Tyr, D-Nal) deviated in the beginning on average by 20% from the expected value. Whereas of those amino acids where 7.5 µM were added, deviation was lower as can be seen in Panel B. All amino acids showed a decrease in signal over time by 13 – 43%, except for the outlier L-Cys (60%) which was excluded from the figure, for a clearer representation of data. Amino acids L- and D-Thr were not completely separated with the LC/MS method used. Therefore, the sum of both overlapping peaks were evaluated as a combined L(D)-Thr form.

Effects of linear trend correction

The QC samples indicated in linear trend over time. Since standards were measured multiple times the decrease could be quantified according to these measurements. Calculated standard concentrations obtained from the three measurements were used to quantify the relative decrease for each amino acid. By plotting the ratio of measured to known concentration c_m/c_k[-] over time, the resultant linear trend is shown for each amino acid analysed in Figure 5.

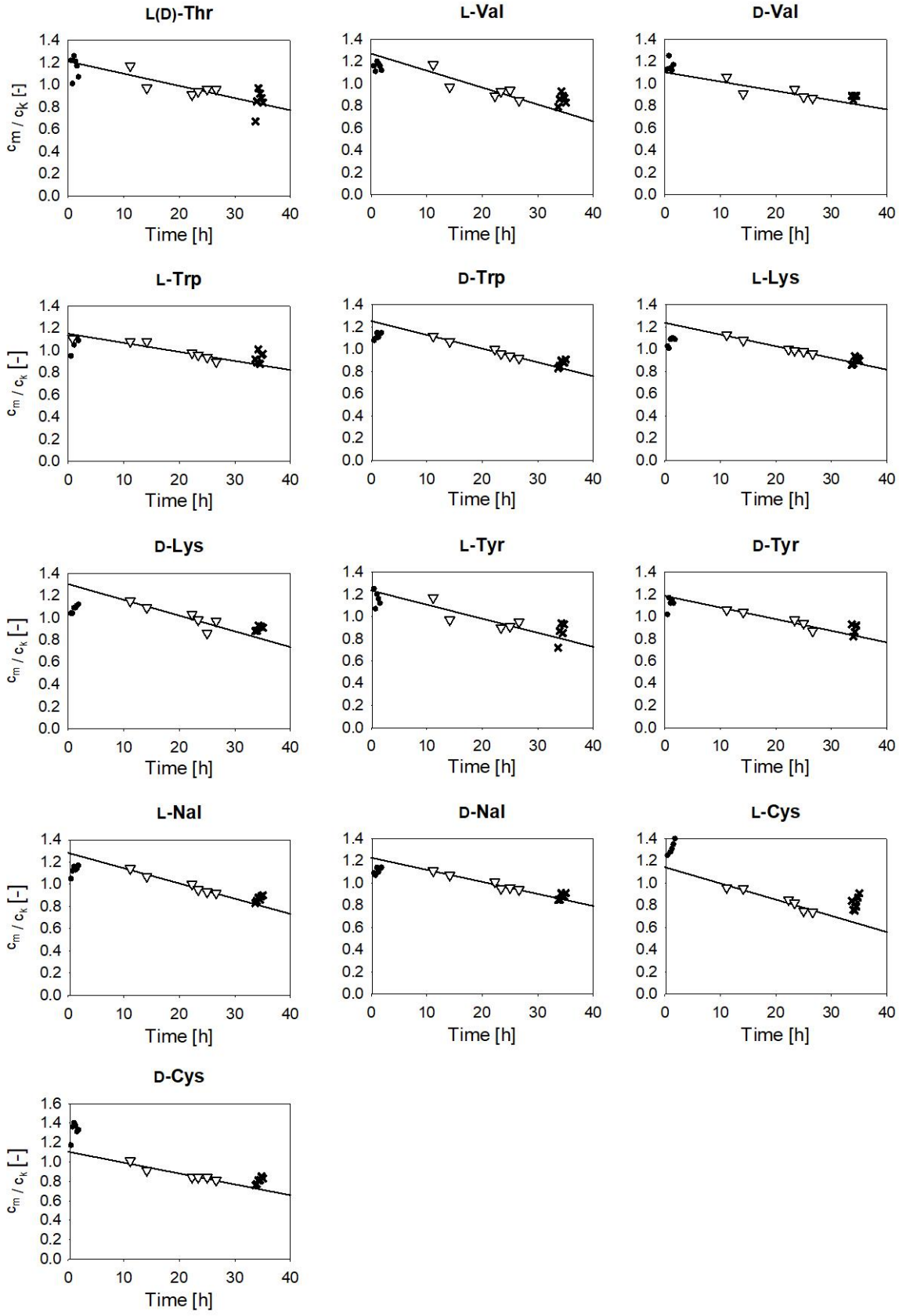


Figure 5: Time-dependent trends of amino acid signals obtained for Lanreotide amino acids. Black dots indicate measurement in the beginning, empty triangles indicate globally randomised measurement between samples, black crosses indicate measurement of standards in the end. c_m/c_k [-] refers to the ratio of measured (c_m) to known concentration (c_k) in standards, respectively.

Table 12: Regression parameters of standards used for linear trend correction of sample values. $k\frac{c_m/c_k}{time}$ [h^{-1}] and $d\ c_m/c_k$ [-] refer to slopes and intercepts of measured (c_m) to known concentrations (c_k) over time, used for linear trend correction. Abbreviations: Err_k, standard error of slope k. R^2 correlation coefficient.

Lanreotide	$k\frac{c_m/c_k}{time}$ [h^{-1}]		Err_k	$d\ c_m/c_k$ [-]	R^2
L(D) -Thr	$-9.10*10$	±	$1.47*10^{-3}$	1.17	0.704
L-Val	$-1.59*10^{-2}$	±	$8.85*10^{-3}$	1.30	0.871
D-Val	$-8.87*10^{-3}$	±	$1.05*10^{-3}$	1.15	0.856
L-Trp	$-4.41*10^{-3}$	±	$9.02*10^{-4}$	1.08	0.599
D-Trp	$-7.55*10^{-3}$	±	$5.56*10^{-4}$	1.14	0.920
L-Lys	$-5.22*10^{-3}$	±	$7.42*10^{-4}$	1.10	0.756
D-Lys	$-5.60*10^{-3}$	±	$8.79*10^{-4}$	1.10	0.717
L-Tyr	$-9.20*10^{-3}$	±	$1.40*10^{-3}$	1.16	0.773
D-Tyr	$-7.36*10^{-3}$	±	$9.09*10^{-4}$	1.13	0.845
L-Nal	$-8.00*10^{-3}$	±	$6.53*10^{-4}$	1.15	0.904
D-Nal	$-7.02*10^{-3}$	±	$5.31*10^{-4}$	1.13	0.916
L-Cys	$-1.59*10^{-2}$	±	$1.86*10^{-3}$	1.30	0.821
D-Cys	$-1.57*10^{-2}$	±	$1.57*10^{-3}$	1.29	0.862

With slope $k\frac{c_m/c_k}{time}$ [h^{-1}] and intercept $d\ c_m/c_k$ [-] obtained from each plot, listed in Table 12, corrected concentrations for standards were calculated according to Equation X for each amino acid. The effect of trend correction is exemplarily shown for L-Val in Figure 6. It can be seen that both, accuracy (slope value should be close to 1) was improved and scattering (reflected by the correlation coefficient R^2 close to 1 and a small value for Err_k) was significantly reduced. The complete set of trend correction is listed in Table 13.

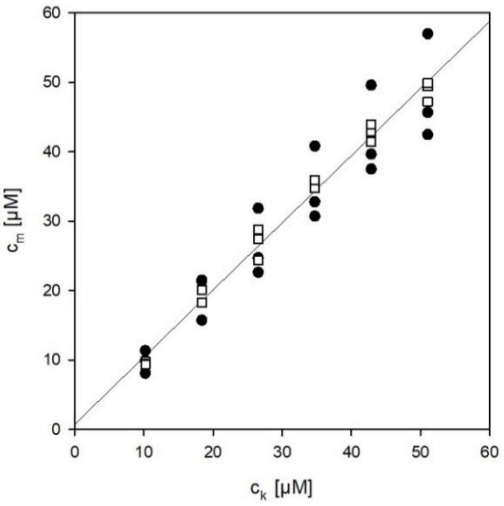


Figure 6: Trend correction illustrated with the example of L-Val. Filled dots – before trend correction. Empty squares – after trend correction. Solid line indicates linear regression after trend correction. C_m refers to measured concentration, c_k refers to known concentration of each standard.

Table 13: Regression parameters of Lanreotide standards before and after correction. $\frac{c_m}{c_k}$ refers to measured (c_m) and known (c_k) concentrations. $\frac{c_{corr}}{c_k}$ refers to corrected standard values (c_{corr}) plotted over known (c_k) standard concentrations. Abbreviations: Err_k, error of slope k. R^2 correlation coefficient.

Lan	Before linear trend correction				After linear trend correction			
AA	$k\frac{c_m}{c_k}$ [µM]	Err_k	$d\frac{c_m}{c_k}$ [µM]	R^2	$k\frac{c_{corr}}{c_k}$ [µM]	Err_k	$d\frac{c_{corr}}{c_k}$ [µM]	R^2
L(D)-Thr	0.95 ± 0.08		2.02	0.905	0.98 ± 0.03		1.21	0.982
L-Val	0.94 ± 0.08		1.28	0.901	0.97 ± 0.02		0.72	0.990
D-Val	1.02 ± 0.09		-0.15	0.918	1.01 ± 0.03		-0.04	0.992
L-Trp	1.01 ± 0.04		-0.13	0.970	1.03 ± 0.02		-0.21	0.992
D-Trp	1.01 ± 0.06		-0.23	0.940	1.03 ± 0.01		-0.69	0.998
L-Lys	1.00 ± 0.05		0.00	0.967	1.01 ± 0.01		-0.29	0.997
D-Lys	1.01 ± 0.05		-0.10	0.957	1.02 ± 0.02		-0.18	0.992
L-Tyr	0.97 ± 0.09		0.87	0.901	1.01 ± 0.04		0.04	0.979
D-Tyr	1.03 ± 0.08		-0.49	0.939	1.02 ± 0.02		-0.31	0.993
L-Nal	1.01 ± 0.07		-0.10	0.931	1.03 ± 0.01		-0.24	0.997
D-Nal	1.01 ± 0.06		-0.18	0.947	1.03 ± 0.01		-0.62	0.998
L-Cys	1.04 ± 0.15		-2.51	0.751	1.02 ± 0.07		-2.34	0.923
D-Cys	1.00 ± 0.14		0.00	0.755	1.05 ± 0.05		-0.94	0.960

Slope k and correlation coefficient R^2 were brought closer to 1. Furthermore, the standard error of slope (Err_k) which indicates scattering of values, could be decreased. The correction resulted in an increase of the correlation coefficient R^2 towards 1. The strongest effects were noticeable for L-Val and D-Cys, with an increase of k values on average by 4 ± 1 %, reduction of standard error by 58 ± 6 % and increase of R^2 towards one by 15 ± 8 %. These data indicated, that the correction could improve the quality of the calibration. Thus, the correction was applied to all values obtained from biological samples, with the respective corrected regression parameters of each amino acid.

Effects of phosphate correction

Previous works at JOANNEUM RESEARCH showed that phosphate in samples might interfere with the LC/MS methodology, resulting in a deterioration of signals [29]. In literature, ion suppression and space charge effects were suggested as contributors to this phenomenon [24]. To investigate whether this phosphate concentration influenced the measurement, QC3, was prepared in 3 different KH_2PO_4 concentrations. The maximum KH_2PO_4 concentration in the 2nd LC/MS measurement was 5.0 mM in undiluted samples. Therefore, the prepared QC3 samples had a concentration of 0, 1, 2.5 and 5 mM KH_2PO_4. These QC samples were used to determine

for which amino acids a phosphate correction was required. The decision was made according to the criterion described in Section 3.9.2. Table 14 summarises, for which amino acids and KH_2PO_4 concentrations the correction was applied, respectively. Values obtained from amino acids D-Tyr, D-Cys, D-Trp and L-Lys were corrected. L-Cys, L-Trp and D-Lys did meet the criterion, so no correction was applied. For the remaining amino acids, phosphate correction was carried out for either one or two phosphate concentrations according to Table 14. To conclude, all biological samples that were previously corrected for the linear trend, where subsequently corrected for phosphate, depending on each amino acid and the respective dilutions made.

Table 14: Phosphate corrections as carried out for amino acids of Lanreotide. Ticks (✓) indicate that the correction was applied as described. Abbreviations: D, sample dilution.

KH_2PO_4 [mM]	D[a]	L-Tyr	L-Val	L-Cys	L-Trp	D-Tyr	D-Val	L-Nal	D-Cys	D-Trp	D-Nal	L-Lys	D-Lys
1.0	1:5	✓	-	-	-	✓	✓	-	✓	✓	-	✓	-
2.5	1:2	✓	-	-	-	✓	-	✓	✓	✓	-	✓	-
5.0	1:1	-	✓	-	-	✓	-	✓	✓	✓	✓	✓	-

[a] Listed dilutions contained KH_2PO_4 in the three concentrations (1.0, 2.5, 5.0), corresponding to KH_2PO_4 concentrations in QC3. Samples were diluted in dH_2O to obtain indicated KH_2PO_4 concentrations.

4.3.3 3rd LC/MS measurement

Similar to the 2nd measurement, one QC sample (LD-QC) was injected seven times throughout the measurement. Standards were measured in the beginning and in the end. The Xcalibur data processing step was carried out successfully for Lanreotide standards measured with Method A. One exception was the end measurement of Std 4, which needed to be excluded for all amino acids. Yet, for Icatibant standards measured with Method A, numerous outliers were noted during the Xcalibur data processing step. Also, the outliers did not affect a single standard repeatedly, or the measurement in the beginning or in the end but rather implied a random pattern. The only noticeable trend was concentration-related, namely higher concentrations were affected with more outliers. Table 15 depicts which standard signals were excluded (# Std. excluded begin/end), due to deviation of linearity.

Table 15: Standards excluded from 3rd LC/MS measurement. Excluded begin/end refers to standards that were excluded from the measurement of standards in the beginning or in the end. Method refers to LC/MS measurement Method A or B. Abbreviations: AA, amino acids; Std., standard; Lan, Lanreotide; Ica, Icatibant; R^2 correlation coefficient after removal of outliers.

Lan AA	Method	# Std. excluded begin	# Std. excluded end	Std. used in total	R^2
L-Thr	A	-	-	14	0.981
D-Thr	A	-	4	13	0.994
L-Val	A	-	-	14	0.990
D-Val	A	-	4	13	0.995
L-Trp	A	-	-	14	0.986
D-Trp	A	-	4	13	0.996
L-Lys	A	-	-	14	0.999
D-Lys	A	-	4	13	0.995
L-Tyr	A	-	-	14	0.991
D-Tyr	A	-	4	13	0.997
L-Nal	A	-	-	14	0.993
D-Nal	A	-	4	13	0.998
L-Cys	A	-	-	14	0.969
D-Cys	A	4, 7	-	12	0.974
Ica AA		**# Std. excluded begin**	**# Std. excluded end**	**Std. used in total**	**R^2**
L-Pro	B	1,7	1,2	10	0.985
D-Pro	B	3	3	12	0.964
D-Hyp	B	-	1,2,3	11	0.973
L-Hyp	B	2	-	12	0.974
L-Oic	B	-	1,2,3	12	0.970
D-Oic	B	-	-	14	0.957
L-Ser	A	1, 3,4	2,4	9	0.991
D-Ser	A	1,4	1,3,4	9	0.931
L-Thi	A	1,4	-	10	0.962
D-Thi	A	1,4	1,2,3,4	9	0.936
L-Tic	A	1,4	2,4	10	0.968
D-Tic	A	1,4	1,2,3	9	0.917
L-Arg	A	1,4	2,4	10	0.958
D-Arg	A	1,4	1,2,3,4,5,6,7	5	0.948
Gly	A	1,4	2,4	9	0.963

After removal of outliers according to Table 15, Xcalibur data processing step was continued (by applying linear regression to the remaining signals) and acquired molar units of standards were analysed further. By that, all amino could be calibrated with similar quality to the 1st and 2nd measurement (for R^2 and calibration range see appendix Table 21) Since the Lanreotide measurement was less problematic, values obtained from Icatibant standards are displayed in Figure 7. In each plot the relationship of measured (c_m) to known concentrations (c_k) for Icatibant amino acids measured with Method A and B is illustrated. This was done to evaluate differences between the two methods.

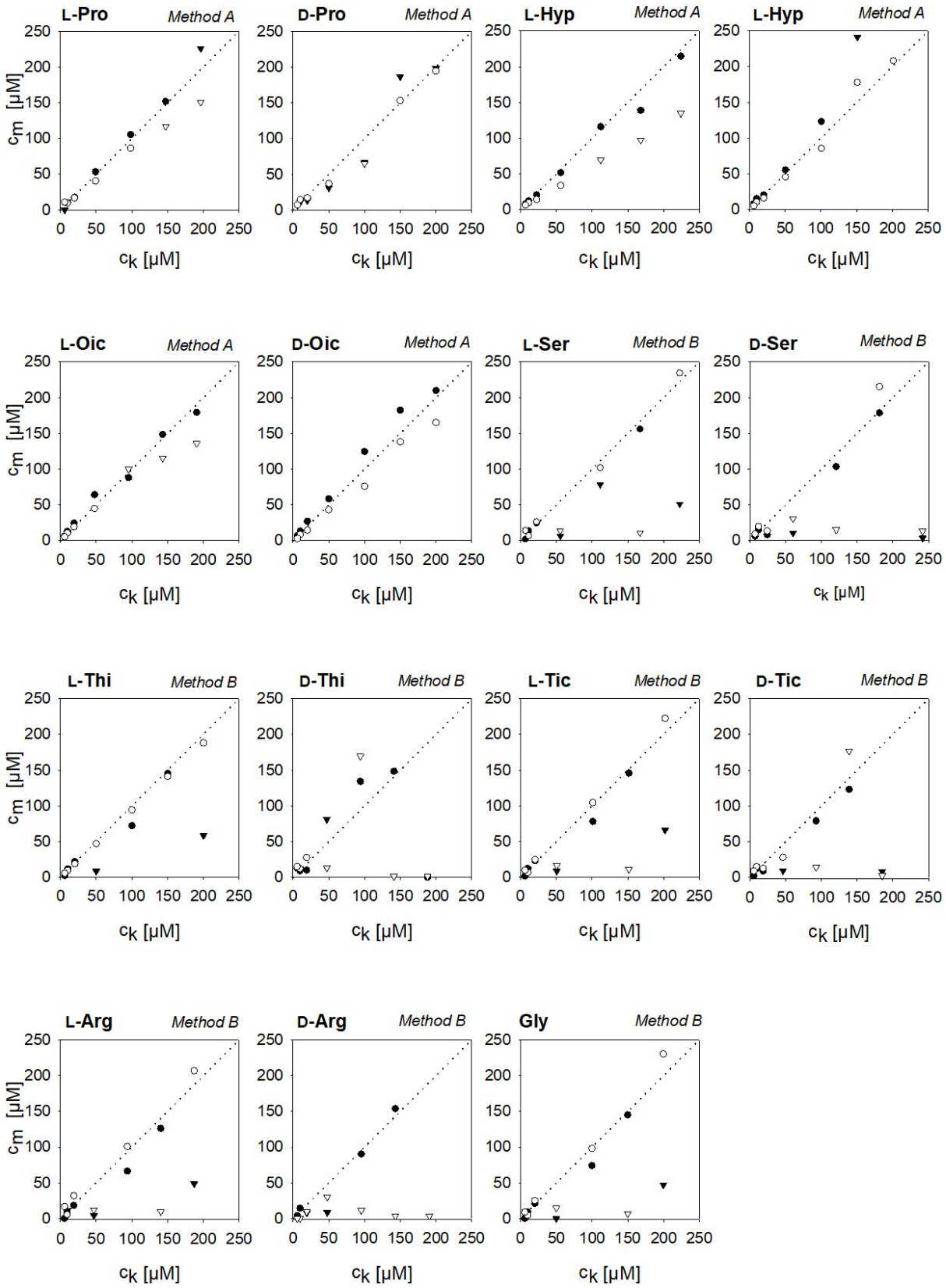


Figure 7: Comparison of known concentrations (c_k) and measured concentrations (c_k), obtained for Icatibant, measured with Method A and B in the 3rd LC/MS measurement. Black filled dots indicate measurement of standards at the beginning - black filled triangles indicate outliers; empty dots indicate measurement of standards in the end, empty triangles indicate outliers. Dotted line represents an optimal relationship of known to measured concentration.

Regarding Figure 7, those amino acids measured with Method A (L/D-Pro, L/D-Hyp, L/D-Oic) showed acceptable linearity with R^2 similar to those of the 1st LC/MS measurement ($R^2 > 0.98$). However, for amino acid standards measured with Method B, significantly more outliers (e.g. 5 out of 14 standards of L/D-Ser, D-Thi, D-Tic, Gly) were observed. With these two measurements sets of standards, calibration was only possible with a reduced number of standards for each amino acid. Values for biological samples determined according to standards from Method B could be afflicted with an error, as similar amounts of outliers, representing wrong amounts of amino acids were expected. For example, the complete set of D-Arg standards measured towards the end had to be excluded. Subsequently, D-Arg values of biological samples obtained towards the end of the measurement had to be removed likewise. Hence, some values in this measurement were not determined by 4, but less replicates.

Correlation coefficients R^2 of Icatibant standards were on average 4% lower than 1, compared to 1% in Lanreotide standards, indicating that scattering of standard values was higher for amino acids measured with Method B.

However, the LD-QCs gave more insights into changing behaviour of the LC/MS measurement signals over time, as illustrated in Figure 8. LD-QCs were prepared in 5.0 mM PB, pH 7.0 from standard 3, containing a final concentration of 25 µM for each amino acid. Figure 8 shows concentrations obtained at the 7 injection timepoints of LD-QCs.

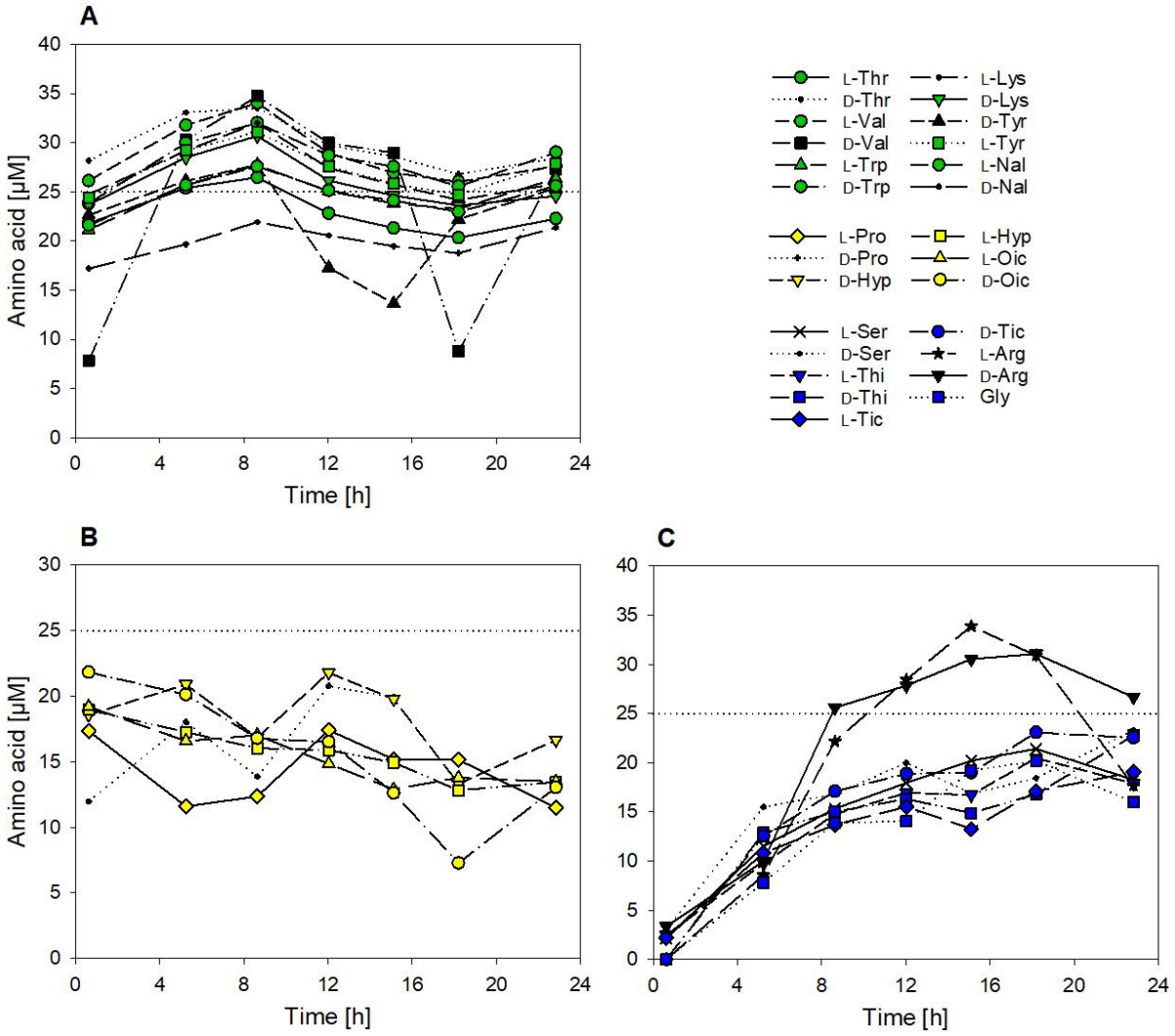


Figure 8: Amino acids of LD-QCs of Lanreotide (panel A) and Icatibant (panel B, C), measured over time. Amino acid of panel A and B were measured with Method A, amino acids of panel C were measured with Method B. Reference lines indicate known concentration (25 µM). Timepoints of measurements were 0.6, 5.2, 8.3, 12.0, 15.1, 18.2, 22.8 h.

Panel A supports previously reported results from standards, indicating that a clear linear trend over time was not noted. A rather wavelike measurement pattern was observed for all amino acids except for D-Val and D-Tyr. Each of these amino acids showed two deviating values (D-Val: 0.6 h, 7 µM; 15.1 h, 8 µM, D-Tyr: 12.0 h, 17 µM; 18.2 h, 13 µM). A slight peak after 8.3 h was observable (28 ± 5 µM), where all values were higher than expected. However, values levelled off at 24 ± 6 µM in the last timepoint. As these deviations over time were considered as relatively small, averages were built from values obtained for samples. Panel B illustrates that amino acid levels of Icatibant contained in LD-QCs, decreased over time but were consistently below the expected concentration of 25 µM (first timepoint: 18 ± 3 µM, last timepoint: 14 ± 5 µM). Trend correction as described in Section 3.9.2 was carried out for all values obtained. The

effect of the linear trend correction was similar to the 2nd measurement. Concerning standards, an improvement of R^2 and k towards 1 and a reduction of Err_k was achieved. Corresponding data can be found in the appendix, Table 22. In panel C a steady increase of values from 0 – 12 h was observed for most amino acids measured. Still, values of the majority of amino acids remained significantly below the reference line of 25 µM. Only L- and D-Arg reached 28 and 27 µM at timepoint 12.0 h. After this timepoint values for all amino acids were relatively stable, resultant in an average scattering of ± 2 µM. Only L-Arg decreased from 30 to 17 µM from timepoint 18.2 – 22.8 h. In biological samples, values measured before 8 h were excluded from data evaluation as these were expected to be significantly lower than the actual values, as seen from LD-QCs in panel C. As a consequence, averages for samples were built from remaining (less) datapoints only if values fit well together.

Icatibant standards measured with Method B (see Table 15 and Figure 7) were compared to corresponding LD-QC results shown in Figure 8 panel C. The Icatibant standard set in the beginning was measured within 3 h. According to LD-QCs, all standards measured before 8 h runtime would be expected at least a third lower, than the actual amount applied (25 µM). Accordingly, the standard set measured in the end would better fit the expected calibration. However, these assumptions do not the fit results from Icatibant amino acid standards measured with Method B (L/D-Ser, L/D-Thi, L/D-Tic, L/D-Arg, Gly) from Figure 8. Based on these irregularities, a more elaborate correction of values was not considered as reasonable. Reasons for this rather random failure of measurements are so far unknown, but are discussed in Section 5.2.

Additionally, L-and D-standards were measured separately in this measurement, to verify chiral purity of each amino acid deriving from the original containers. In none of the respective standards, peak signals of variants of L- or D- forms were found, so chiral impurities from the containers could be disproved. This finding was important considering experiments with the AA-mix of peptides investigating stability of free amino acids under different incubation conditions.

4.4 Acid hydrolysis

Stability of free amino acids, estimation of amino acid content by OPA assay and acid hydrolysis behaviour over time is evaluated in this section. Corresponding data was acquired in the course of the 2nd and the 3rd LC/MS measurement.

4.4.1 Amino acid and protease stability

An AA-mix representing each peptide was used, to investigate stability of free amino acids in buffered solution (5.0 mM BP, pH 7.0) and under acidic (6 M HCl, 160°C, 30 min) or enzymatic (NAP-purified Pronase or Flavourzyme, 5.0 mM PB, pH 7.0, 50° C, 24 h) hydrolysis conditions. Additionally, NAP-column purified enzymes incubated in a buffered solution, as in an enzymatic hydrolysis served as stability controls for enzymes Figure 9 illustrates LC/MS results of amino acid mixtures representing Lanreotide (panel A) and Icatibant (panel B).

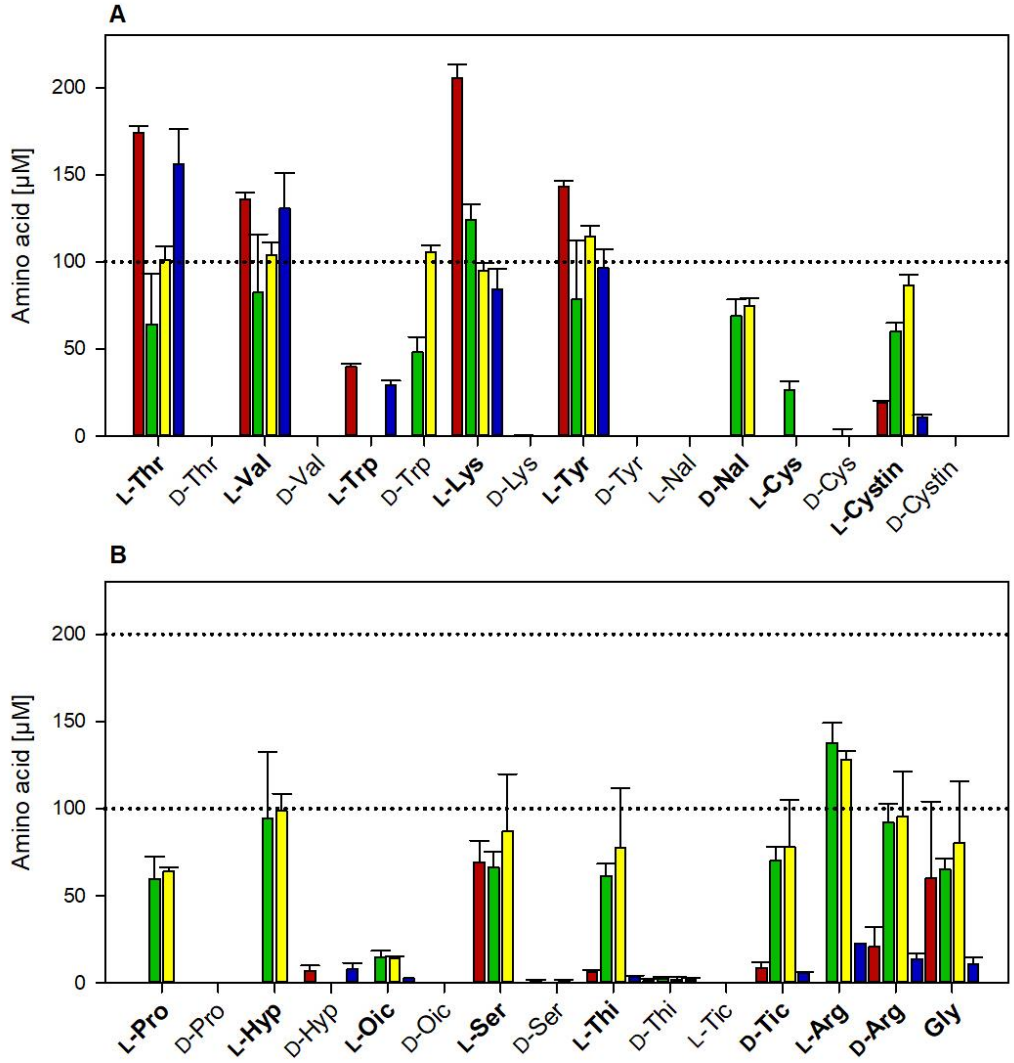


Figure 9: Results from stability experiment of AA-Mix and proteases. Panel A: Lanreotide panel B: Icatibant. Dotted line: expected amount of amino acids (100 µM) applied to each reaction. For L-Arg 200 µM were expected. Bold written amino acids occur naturally in each peptide. Red bars: Flavourzyme; blue bars: Pronase; green bars: AA mix under acidic hydrolysis conditions; yellow bars: AA mix under enzymatic hydrolysis conditions.

Each mix contained 100 mM of each amino acid respectively, except for L-Arg where 200 µM were added to the mixture, because L-Arg occurs twice in Icatibant. To account for the disulphide bridge between the two L-Cys in Lanreotide, Cystin was added to the AA-mix. All amino acids applied in each AA-mix were detectable, as illustrated in Figure 9. In Panel A, amounts for Lanreotide AA-mix under acidic treatment measured, were on average one forth lower than the actual amount present in the mixture, except for L-Lys (125 ± 9 µM). The amount of amino acids measured from Icatibant AA-Mix in buffer (panel B) corresponded to the expected 100 µM, except for D-Nal (74 ± 4 µM) and Cystin (86 ± 5 µM). For both AA-Mix preparations of Icatibant (Panel B) amounts of amino acids found were a third lower than expected, except for L-Oic (2 ± 0.3 µM). Degradation of amino acids over time or measuring lower signals than expected with the LC/MS methodology could be taken into consideration, but the true nature of these losses is not known.

Chiral variant amino acids where either not detectable or occurred in extremely low to negligible concentrations, mostly below the lowest calibration point. In AA-Mix of both peptides D-Thi (2 ± 0.3 µM) and D-Ser (1 ± 0.8 µM) were found, in both enzyme fractions artificial amino acids L-Thi (4 ± 1 µM), D-Tic (7 ± 2 µM), D-Hyp (7 ± 0.6 µM) and chiral variants of natural amino acid D-Ser (1 ± 0.3 µM) and D-Arg (17 ± 5 µM) were detectable. These values could be explained by inaccuracy of the measurement methodology. The findings underline that neither under enzymatic nor under acidic hydrolysis conditions free amino acids were converted in significant amounts into their chiral amino acid variant.

Another observation in Figure 9 was that in samples of solely proteases incubated in buffer, amino acids were detected. Red and blue bars of Flavourzyme and Pronase respectively, illustrate that statistically significant amounts of L-Thr, L-Val, L-Trp, L-Lys and L-Tyr were found. These amino acids are further referred to as autolysis amino acids. Furthermore, only significant amounts of amino acids of the L-form were found which is in accordance with autolysis of proteases according to literature [100]. Comparison of the production of autolysis amino acids in different approaches with purified and non-purified enzymes is discussed later on.

In addition to the LC/MS measurement, ultrafiltrates of AA-mix of each peptide and filtrates of the two proteases treated under enzymatic hydrolysis conditions, were analysed with OPA-assay. This was done to analyse the amount of primary NH_2 groups, in this case corresponding to the total amount of amino acids, measured with OPA assay.

Figure 10 illustrates total amino acid content in AA-mix of Lanreotide (black bars) and Icatibant (grey bars). Data was acquired by converting absorbance values to molar units by using extinction coefficients of amino acids specific for Lanreotide and Icatibant, explained in Section 4.2. For Lanreotide, and Icatibant 0.6 mM were detected, although they consist of 8 and 10 amino acids, respectively. The fact that 5 out of 9 amino acids of Icatibant could not be complexed with OPA due to secondary amines, might explain this finding.

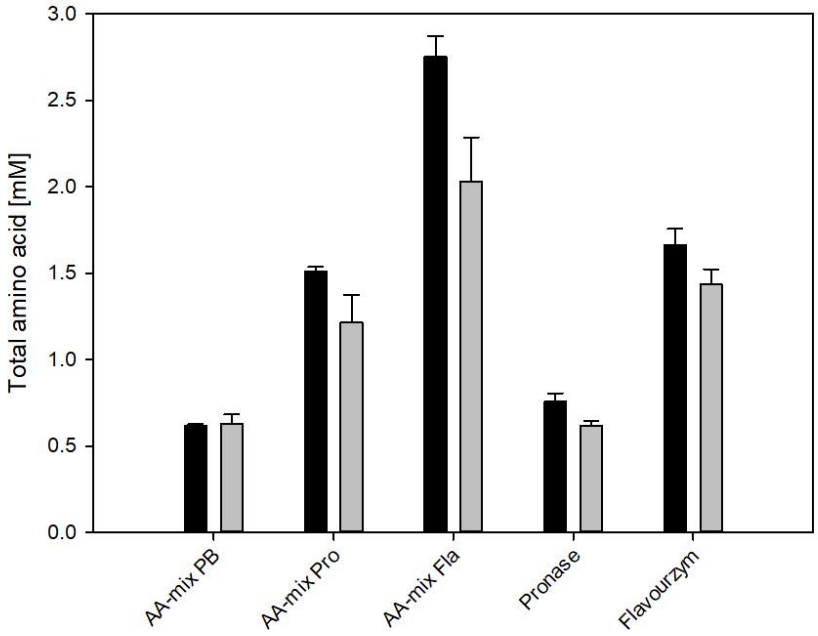


Figure 10: OPA-assay analysis of AA-mix and enzymes treated under enzymatic hydrolysis conditions. Black bars: Lanreotide, grey bars: Icatibant. Abbreviations: Pro, Pronase; Fla, Flavourzyme.

Although both enzymes were purified with NAP columns prior to usage, a high portion of autolysis amino acids was observed. Flavourzyme produced approximately the double amount compared to Pronase. As a consequence, each AA-mix incubated with the enzymes showed increased amounts of free amino acids. Values of 1.5 ± 0.02 mM and 1.2 ± 0.1 mM were obtained for Lanreotide and Icatibant AA-mix incubation with Pronase, while 2.7 ± 0.1 mM and 2.0 ± 0.2 mM were detected for Flavourzyme. By summing up L-amino acids found in enzymes from LC/MS measurement of the 3rd measurement (Method A and B) from stability studies, values of 558 ± 33 µM and 846 ± 46 µM were found for Pronase and Flavourzyme. Compared to OPA results, OPA values of Flavourzyme are twice as high compared to LC/MS results, whereas those of Pronase fit the OPA results well.

4.4.2 Time course of acid hydrolysis

In the standard procedure of JOANNEUM RESEARCH HEALTH, acid hydrolysis of peptides is conducted for 30 min. The following time course experiments should on the one hand show, whether shorter or longer incubations (10 – 95 min) would have a beneficial effect on the total degree of hydrolysis of a peptide, and on the other hand whether racemisation is occurring in a time-dependent manner.

Figure 11 depicts results from acid hydrolysis time course experiments of Lanreotide (panel A, B) and Icatibant (panel C, D). After a reaction time of 10 minutes all amino acids of Lanreotide were already found in hydrolysates (panel A). Only a slight increase of concentrations measured could be recognised from 10 – 95min. For example, a hydrolysis rate of 38 % was seen for D-Trp after 10 min, which increased to 43 % after 90 min. In other words, after 10 min already 89% of total hydrolysis for D-Trp was already reached, referring to the value at 90 min as 100%.

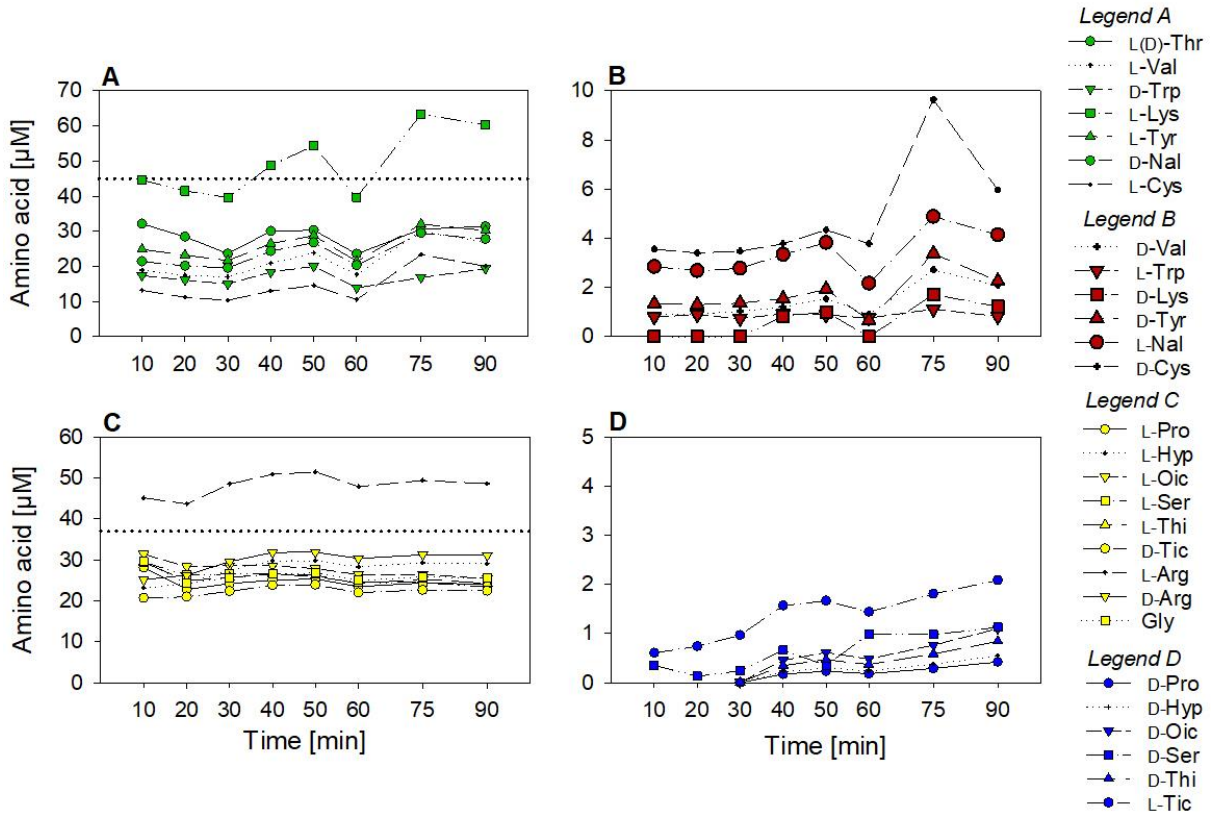


Figure 11: Time course of Lanreotide and Icatibant acid hydrolysis. Timepoints of hydrolysis as depicted in x-axis. Panel A and B: Amino acids of Lanreotide (A) with corresponding chiral variants (B). Panel C and D: Amino acids of Icatibant (C) with corresponding chiral variants (D). Reference bars indicate expected values: Icatibant: 37 µM, except for L-Arg 74 µM; Lanreotide 45 µM except for L-Cys 90 µM.

The same applied for L-Tyr, L-Val, L-Cys and D-Nal for which a hydrolysis rate of 55%, 42%, 29% and 47% was determined after 10 min, respectively. After 90 min reaction time they showed a degree of hydrolysis of 67%, 60%, 44% and 61%, respectively. Complete hydrolysis was observed in neither of the peptides, but on average a degree of hydrolysis of 47 ± 13 % was seen after 10 min which increased to 59 ± 11 % after 90 min. Due to this slight increase by only 12% from 10 – 90 min, a fast proceeding of the acid hydrolysis reaction can be assumed. All values dropped from timepoint 50 min to 60 min (e.g. L-Lys 27%), which could have been caused by measurement inaccuracies. In general, the degree of hydrolysis is defined as the proportion of free amino acids found in a protein hydrolysate. Since different amounts of each amino acid were hydrolysed, the average degree of hydrolysis for Lanreotide was affected with a high standard deviation. Thus, a value of 54 ± 28% was determined.

Panel B, illustrates that chiral amino acid variants were found in hydrolysates of Lanreotide after an acid hydrolysis. Similar to Panel A, all chiral variants (L-Trp, D-Tyr, D-Val, L-Nal, D-Cys) except for D-Lys were already present after 10 min. On average all of these amino acids were found at concentrations below 2 µM, except for D-Cys, which gave an average value of 4 ± 2 µM and L-Nal with an average of 3 ± 0.8 µM. The highest response of D-Cys after 75 min (9 µM) might have derived from measurement inaccuracies. Again, 68% of the 90 min value e.g., for D-Cys were already achieved after 10 min which suggested a rather fast reaction. For Lanreotide an average degree of racemisation of 4% can therefore be estimated.

Results from Icatibant in panel C were consistent with findings from Lanreotide. Nevertheless, values scattered less. After 10 min of incubation 92%, 80% and 79% of total hydrolysis rates were already achieved for L-Arg, D-Arg and L-Hyp, respectively. Neither a significant increase of concentrations over time, nor complete hydrolysis of the peptide was observed. On average, a total degree of hydrolysis of 68 ± 7% was determined for Icatibant.

In panel D all values obtained were below the calibration range, however a slight increase over time of L-Tic up to 2 µM after 90 min was noted. This amount equals to 4% D-Tic being racemised to the chiral variant. On average, racemisation of Icatibant amounted 1% These values have to be interpreted with great caution, as they were all found below the lowest calibration point.

4.5 Enzymatic hydrolysis

In this chapter feasibility of enzymatic hydrolysis reactions with the two proteases applied is evaluated. After conducting the experiments as described in Section 3.5.1, concentration of each amino acid investigated was determined by LC/MS and total amino acid content was determined photometrically by OPA-assay. By changing values of parameters like reaction time, amount of enzyme and substrate offered, as well as quality of protease (purified or non-purified), closer insights into pharmaceutical peptide analysis by proteases were attempted to be gained.

4.5.1 Enzymatic hydrolysis with non-purified enzyme

In this study 10 U of each protease were added to 1.0 mM of peptide and samples were withdrawn after 0 and 24 h. Specific activity of Flavourzyme was 2.19 U/mg and for Pronase 19.99 U/mg prior to usage. Results were obtained by LC/MS analysis (1^{st} hydrolysis) after 24h of peptide hydrolysis. Each sample was measured at two different dilutions (1:20 and 1:40) and samples were diluted with dH_2O. Values shown in Table 16 correspond to averages plus standard deviations, considering both dilutions.

The particularly high standard deviations (e.g. L-Ser, L-Lys, L-Arg) indicated that the two dilutions did not correlate. One reason might be that the concentration of amino acids in samples exceeded the calibration range. As a consequence, data in Table 16 have to be seen as rather rough estimations. Stability of enzymes and peptides during hydrolysis reactions had to be evaluated prior to analysing enzymatic hydrolysis reactions. In an optimal reaction, peptide and enzymes were expected to be stable during the hydrolysis reaction conditions, so amino acids should not be found in significant amounts in controls applied (peptide and enzyme incubated in buffer for 0 – 24 h).

Signals obtained of L-Tic in Icatibant and D-Thr, L-Trp, L-Lys, L-Tyr, D-Tyr and D-Nal in Lanreotide samples were regarded as artefacts of LC/MS measurement inaccuracies, as standard deviations were similarly high as the actual values (see Table 16). Hence, for both enzymes high amounts of naturally occurring – as described previously – autolysis amino acids like L-Lys of L-Trp were detected. As depicted in Tables 16, the effect of autolysis was stronger for Flavourzyme than for Pronase.

To estimate feasibility of enzymatic hydrolysis, the amount of D- and artificial amino acids was analysed. In Icatibant hydrolysis with Flavourzyme D-Tic (47 ± 1 µM), L-Thi (238 ± 21 µM) and D-Nal (272 ± 91 µM) were found. Pronase hydrolysed amino acids D-Tic (51 ± 36 µM), L-Thi (414 ± 37 µM) and D-Nal (91 ± 119 µM).

Based on these artificial amino acids, a degree of hydrolysis between 4 – 27% can be estimated. Natural and L-amino acids cannot be considered to determine the degree of hydrolysis, because for example from 2261 ± 305 µM of L-Trp, only a part could derive from the hydrolysis reaction itself (1 mM peptide initially applied in the reaction) the remaining amount probably originateed from autolysis. In addition, the value is an estimation, because the calibration range (L-Trp: 3 – 15 µM) was exceeded by multiples. The production of chiral variants due to proteases was not observed in this study.

Table 16: Amino acids found in filtrates of enzymatic hydrolysis after 24 h. Values are presented as average ± standard deviation, obtained from dilutions 1:20 and 1:40. Ica/Lan – Fla/Pro refers to enzymatic hydrolysis of peptide with each enzyme. Abbreviations: Ica, Icatibant; Lan, Lanreotide; Fla, Flavourzyme; Pro, Pronase.

Ica AA	Ica [µM]	Fla [µM]	Pro [µM]	Ica – Fla [µM]	Ica – Pro [µM]
L-Ser	0	2925 ± 206	1151 ± 336	3016 ± 213	993 ± 345
L-Thi	0	0	0	238 ± 21	414 ± 37
D-Thi	0	0	0	0	0
L-Tic	5 ± 4	0	0	0	0
D-Tic	0	0	0	47 ± 1	51 ± 36
L-Arg	0	2404 ± 107	1102 ± 355	2476 ± 953	946 ± 390
D-Arg	0	0	0	0	0

Lan AA	Lan [µM]	Fla [µM]	Pro [µM]	Lan – Fla [µM]	Lan – Pro [µM]
L-Thr	0	2685 ± 624	1459 ± 273	2865 ± 622	622 ± 191
D-Thr	19 ± 16	0	0	0	0
L-Val	0	4042 ± 287	1685 ± 338	3951 ± 371	371 ± 429
D-Val	0	0	0	0	0
L-Trp	9 ± 7	2585 ± 126	1384 ± 194	2261 ± 305	305 ± 48
D-Trp	0	0	0	57 ± 24	24 ± 20
L-Lys	17 ± 15	3104 ± 229	948 ± 237	2681 ± 416	416 ± 475
D-Lys	0	0	0	0	0
L-Tyr	20 ± 15	1523 ± 167	1038 ± 211	1422 ± 244	244 ± 344
D-Tyr	14 ± 18	0	0	0	0
L-Nal	0	0	0	4 ± 6	2 ± 1
D-Nal	16 ± 20	0	0	272 ± 91	91 ± 119
D-Cys	0	0	0	0	0

In addition, samples were analysed with the OPA-assay. After incubating enzymes for 24 h, the portion of autolysis amino acids in the enzyme-references was significantly high for both enzymes (Flavourzyme 3.2 ± 0.5 mM, Pronase 1.8 ± 0.3 mM). As hydrolysis samples gave nearly the same values as the enzymes themselves, determining the degree of hydrolysis was not possible. However, in filtrates of the peptides incubated in buffer for 24 h, no absorbance was measured, which indicated, that the peptides were stable during incubation at 50° C.

Based on these results, the following conclusions were drawn: As data obtained from this 1st LC/MS measurement showed high deviations, LC/MS methodology was adjusted and data for the 2nd hydrolysis, gained from the 2nd LC/MS measurement was corrected for a time-dependent linear trend and effects of phosphate as described in Section 3.9.2. Autolysis of proteases took place, which made quantification of hydrolysed amino acids from the peptide and further the determination of degree of hydrolysis difficult. Due to artificial amino acids a degree of hydrolysis by 4 – 27% was estimated. To enable quantification for the next enzymatic hydrolysis, proteases were purified by gel filtration prior to usage. In addition, two different concentrations of Lanreotide (1.0 mM and 0.2 mM) were applied and samples were withdrawn after 0, 3, 6 and 24 h. By subtracting the portion of autolysis amino acids from an enzymatic conversion with a peptide, quantification of degree of hydrolysis should be enabled for more than just artificial amino acids.

4.5.2 Enzymatic hydrolysis of Lanreotide – time course experiment

It was attempted to gain more information about an enzymatic hydrolysis reaction of Lanreotide over time, using 10 U of NAP purified Flavourzyme or Pronase. Hydrolysis reactions were carried out with 0.2 and 1.0 mM Lanreotide, at 50°C and amino acid levels were determined over time at 3, 6 and 24 h. Reference experiments were conducted by incubating peptide and enzymes separately under otherwise identical conditions and additionally to the specified timepoint, samples were taken after 0 h. Resultant LC/MS data were corrected by linear trend and phosphate correction as described in Section 3.9.2. In addition, samples were analysed with the OPA-assay to determine total amino acid content.

Specific activity of Flavourzyme and Pronase was 3.33 U/mg and 12.95 U/mg and was reduced by about 50% after NAP purification to 1.53 U/mg 6.53 U/mg. Amino acid content was reduced by 42% in Pronase an 82% in Flavourzyme. Similar to results obtained in Section 4.5.1, Lanreotide incubated in buffer over 24 h was stable and no free amino acids were detected. The portion of autolysis amino acids was again considerably elevated after 24 h, but the amount of all amino acids found (except for L-Lys) was decreased due to NAP purification. Results are depicted in Figure 11. In both enzymes the same amino acids were found. For Flavourzyme the amount was roughly a third higher, except for L-Lys, of which three times the amount of Pronase was detected.

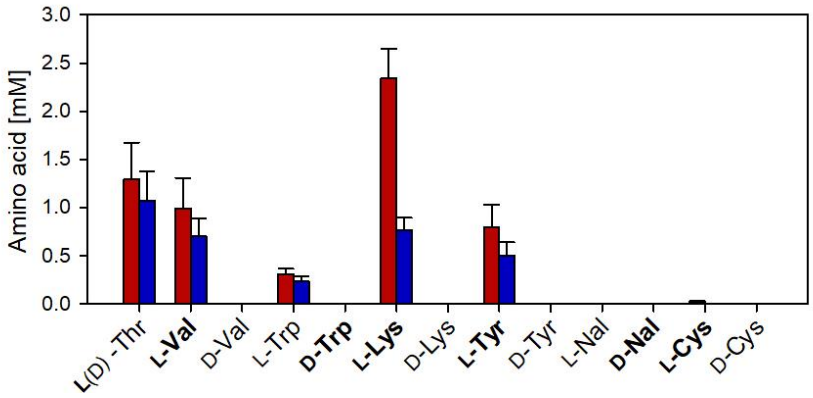


Figure 11: Autolysis amino acids found in enzyme references after 24 h in the course of the 2nd enzymatic hydrolysis. Red bars represent amino acids of Flavourzyme, blue bars represent those of Pronase. Amino acids written in bold letters occur naturally in Lanreotide.

Table 17 compares the portion of autolysis amino acids from the 1st and the 2nd enzymatic hydrolysis obtained after 24 h. Again, values exceeded the calibration range (3 – 50 mM), therefore results represent estimated values. By comparing results from 1st and 2nd hydrolysis (24 h timepoints each), a significant decrease of amino acids liberated was observed. For example, in Flavourzyme the portion of L(D)-Thr, L-Val and L-Trp was reduced by 51%, 75% and 87%, from 1st to 2nd hydrolysis, respectively. In Pronase this applied to 26%, 58% and 83%, respectively. Values for L-Lys were highly increased in both batches (1st hydrolysis: 3.1 mM, 0.9 mM; 2nd hydrolysis: 2.3 mM, 0.7 mM for Flavourzyme and Pronase). In this case, a reduction of 24% and 18% was achieved for Flavourzyme and Pronase, respectively. On average the reduction amounted 1.8 mM for Flavourzyme and 0.5 mM for Pronase. The extent of purification by NAP columns could thereby be shown on a mass spectrometric basis due to LC/MS results in this study. Furthermore, only amino acids that occur naturally in the peptides, were set free and no D- amino acids were detected.

Table 17: Autolysis amino acids found in enzyme references after 24 h in the course of the 1st and the 2nd enzymatic hydrolysis. Abbreviations: AA, amino acids; Lan, Lanreotide; NC, not calibratable.

Lan	1st enzymatic hydrolysis						2nd enzymatic hydrolysis					
AA	Flavourzyme [µM]			Pronase [µM]			Flavourzyme [µM]			Pronase [µM]		
L-Thr	2685	±	624	1459	±	273	1296	±	377	1074	±	305
D-Thr		0			0			NC [a]			NC [a]	
L-Val	4042	±	287	1685	±	338	992	±	314	700	±	191
D-Val		0			0			0			0	
L-Trp	2585	±	126	1384	±	194	312	±	56	232	±	58
D-Trp		0			0			0			0	
L-Lys	3104	±	229	948	±	237	2345	±	306	770	±	127
D-Lys		0			0			0			0	
L-Tyr	1523	±	167	1038	±	211	800	±	234	505	±	134
D-Tyr		0			0			0			0	
L-Nal		0			0			0			0	
D-Nal		0			0			0			0	
L-Cys		0			0		24	±	10		0	
D-Cys	2685	±	624	1459	±	273		0			0	

[a] Thr was evaluated as a combined L(D)-Thr form, which is why no values are given for D-Thr for the 2nd measurement.

Additionally, to the three timepoints where samples of peptide conversions were taken, protease samples in buffer were withdrawn after 0 h of incubation. Amino acids that were found after 0, 3, 6 and 24 h in enzyme controls are illustrated in Figure 12, with results from Flavourzyme in panel A and results from Pronase in panel B. For a better representation of data, scaling is different in both panels.

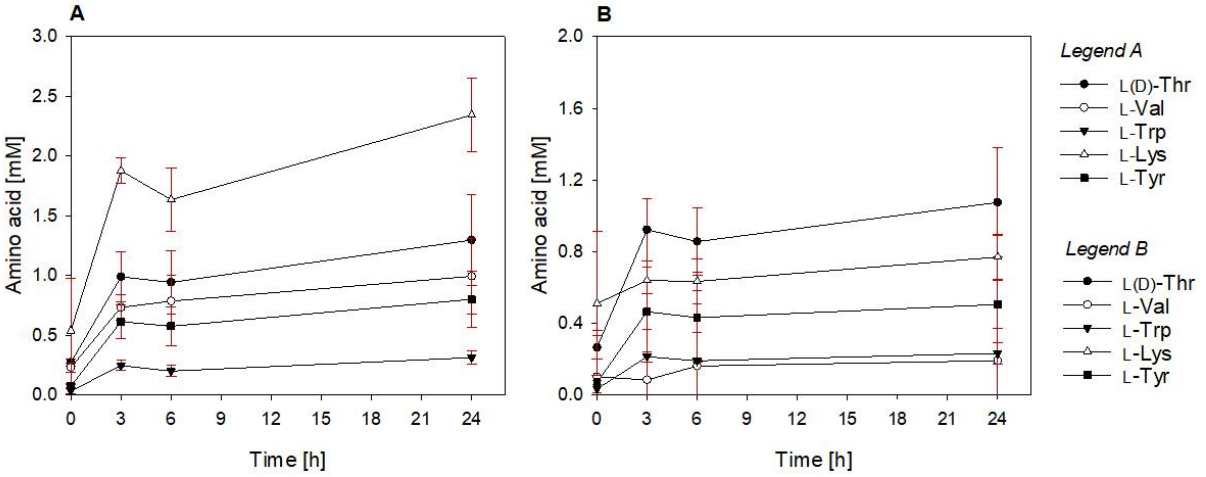


Figure 12: Autolysis amino acids found in enzymes over time. Timepoints were 0, 3, 6 and 24 h. Panel A: Flavourzyme, Panel B: Pronase. Respective error bars are depicted in red.

Amino acid release due to autolysis was fast within the first 3 h and slowed down (e.g. L-Lys, panel A) or even stagnated (e.g. L-Trp, panel A and B) for the residual 18 h, considering values within their respective error bars. Taking Flavourzyme as an example, at timepoint 0 h, 20% L(D)-Thr, 23% L-Val and 10% L(D)-Trp of the 24 h value were already detected. After 3 h, on average 76 ± 2 % of L(D)-Thr, L-Val and L-Trp were produced. In Pronase this value was even higher with 90 ± 4 % for these three amino acids. In Figure 13, time courses of those amino acids with values above the lowest calibration point > 3 µM, are shown. The complete dataset is found in the appendix, Table 23. Note that scaling is different again.

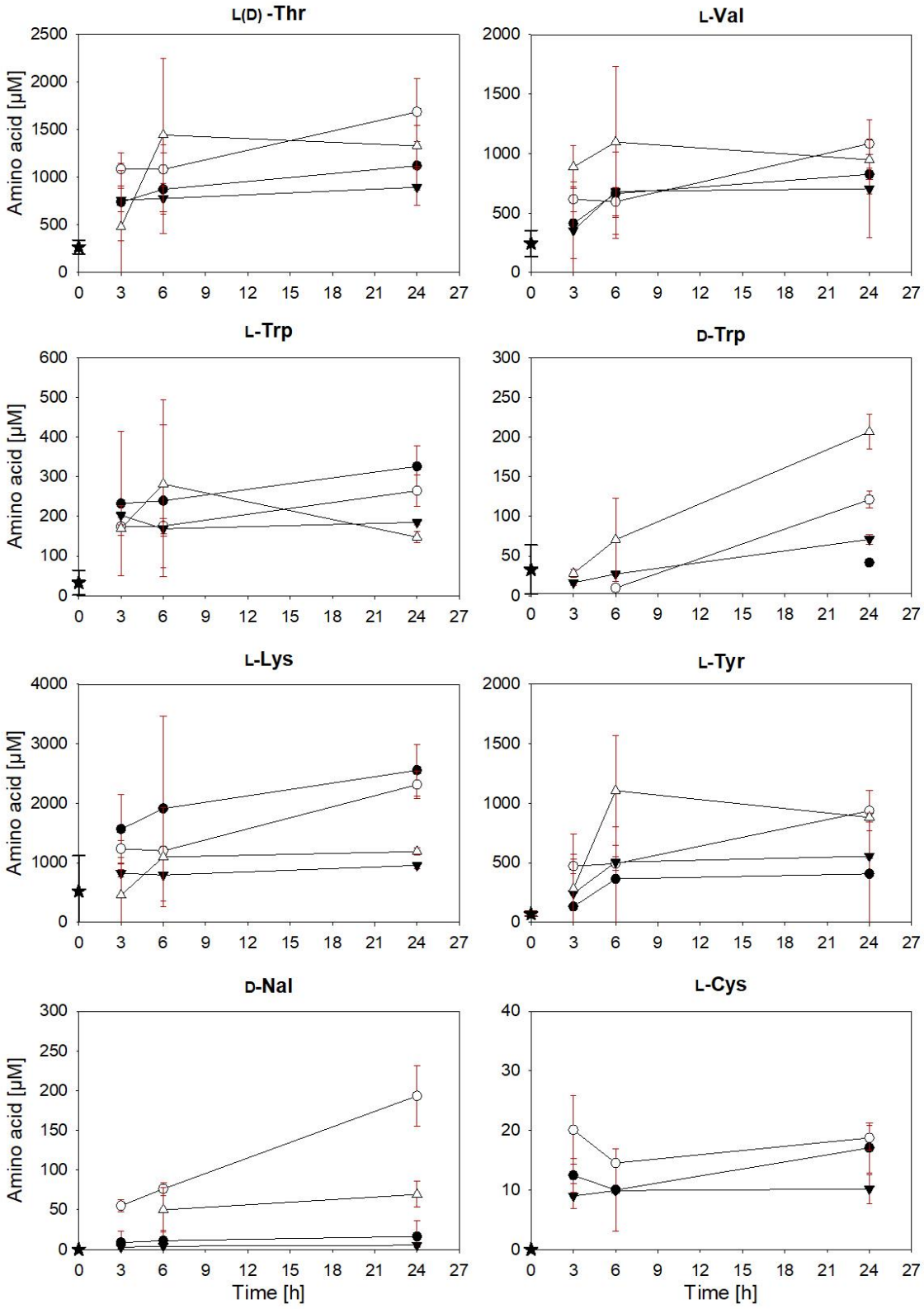


Figure 13: Amino acids Results from time course experiments with Lanreotide. Amino acids which were found in hydrolysates after 3, 6 and 24 h are depicted, except for D-Pro and L-Nal as values obtained were below the calibration range. Vertical red lines indicate error bars. Empty circles: Flavourzyme with 1.0 mM Lanreotide; black filled circles: Flavourzyme 0.2 with mM Lanreotide; empty triangles: Pronase with 1.0 mM Lanreotide; black filled triangles: 0.2 with mM Lanreotide. Stars represent average timepoint 0 values for Flavourzyme and Pronase.

No data was obtained for D-Val, D-Tyr, and D-Cys from 0 to 24 h. After 24 h, 4 µM of D-Pro were observed after 24 h with Pronase and 1.0 mM Lanreotide, similarly, 4 µM L-Nal were detected with Flavourzyme and 0.2 mM Lanreotide. As these values were found at the lowest level of the calibration range, and quantification of LC/MS results remains afflicted with rather high standard these values must be interpreted with great caution. In samples where 1.0 mM Lanreotide was added, more of each respective amino acid than the expected amount was produced, except for L-Trp (Flavourzyme: 295 ± 43 µM, Pronase: 166 ± 26 µM) and D-Nal (Flavourzyme: 193 ± 38 µM, Pronase: 69 ± 16 µM). A significant difference regarding the hydrolysis efficiency of both enzymes was not revealed. However, Flavourzyme released 67% (0.2 mM Lanreotide) and 64% (1.0 mM Lanreotide) more of D-Nal than Pronase. On the contrary Pronase liberated 41% more D-Trp than Flavourzyme from 0.2 mM and 1.0 mM Lanreotide. These findings coincide with the 1st enzymatic hydrolysis were D-Nal and D-Trp were found, and Flavourzyme produced more of these amino acids in both experiments. Given the fact that the majority of L-amino acids liberated derived from autolysis, a conclusion concerning hydrolysis efficiency over time, based on L-amino acids must be treated with caution. But as D-amino acids released could derive from the peptide itself, rough estimations can be made. Values of L(D)-Thr, L-Val, L-Trp, L-Tyr and L-Cys tended to stagnate after 6 h, representing a two-phase behaviour over time. D-Trp was liberated steadily by both enzymes up to 24 h, which indicates a single-phase increase of amino acid. From 24 h Lanreotide-Pronase hydrolysates a D-Trp degree of hydrolysis of 7% and 20% (0.2 mM and 1.0 mM Lanreotide) was estimated. For Lanreotide-Flavourzyme hydrolysates values were 4% and 12%. For D-Nal 0.5% and 7% degree of hydrolysis with Pronase and 1.6% and 19% with Flavourzyme was estimated for 0.2 mM and 1.0 mM Lanreotide, respectively.

Autolysis amino acids were subtracted from hydrolysis reactions, to evaluate whether or not an increase of L-amino acids, deriving doubtlessly from the peptide, was visible over time. Values above the expected amount or below zero were excluded. By subtracting Flavourzyme from hydrolysates 65 µM L-Lys (0.2 mM Lanreotide), 136 µM L-Tyr and 90 µM L-Val (1.0 mM Lanreotide) were found after 24 h. Subtraction of Pronase in corresponding samples resulted in 50 µM L-Tyr, 185 µM L-Lys (0.2 mM Lanreotide), 255 µM L-Thr, 376 µM L-Tyr and 416 µM L-Lys (1.0 mM Lanreotide). For Flavourzyme the average degree of hydrolysis after 24 h for L-amino acids found from the subtraction was 20 ± 14%. For Pronase it was 44 ± 27%. By excluding L-Lys, due to high standard deviations degree hydrolysis of Pronase was 32 ± 8% and 17 ± 14% for Flavourzyme.

Based on these values, determination of total degree of hydrolysis was possible for more amino acids compared to the 1st enzymatic hydrolysis. Namely, for 5 L-amino acids: L(D)-Thr, L-Tyr, L-Lys, L-Val, L-Trp and 2 D-amino acids: D-Trp, D-Nal.

By means of OPA assay, total amino acid content in hydrolysates was determined. Results are depicted in Figure 14. For both enzymes amino acids were already detected at the 0 h timepoint (Flavourzyme 0.3 ± 0.1 mM, Pronase 0.2 ± 0.02 mM). These values increased for both proteases up to 6 h and stagnated afterwards. This over-time behaviour coincides with findings from LC/MS measurements and amino acid content measured, probably refers to autolysis. This contribution makes assessment of actual hydrolysis efficiency difficult. However, in Panel A, peptide hydrolysis conducted by Flavourzyme is visible for the 1.0 mM sample. By subtracting the amount of amino acids from Flavourzyme, from the hydrolysis, a value of 0.44 mM can be determined. For the 0.2 mM sample this subtraction cannot be made, because a negative value would result from it (compare Panel A, empty triangle). Panel B shows that Pronase produced 1.4 mM less autolysis amino acids than Flavourzyme. A likewise subtraction of protease amino acids from the hydrolysate results in 0.21 and 0.09 mM for 1.0 mM and 0.2 mM peptide offered. These values would indicate a degree of hydrolysis of 40% for Flavourzyme and 21% and 45% for Pronase.

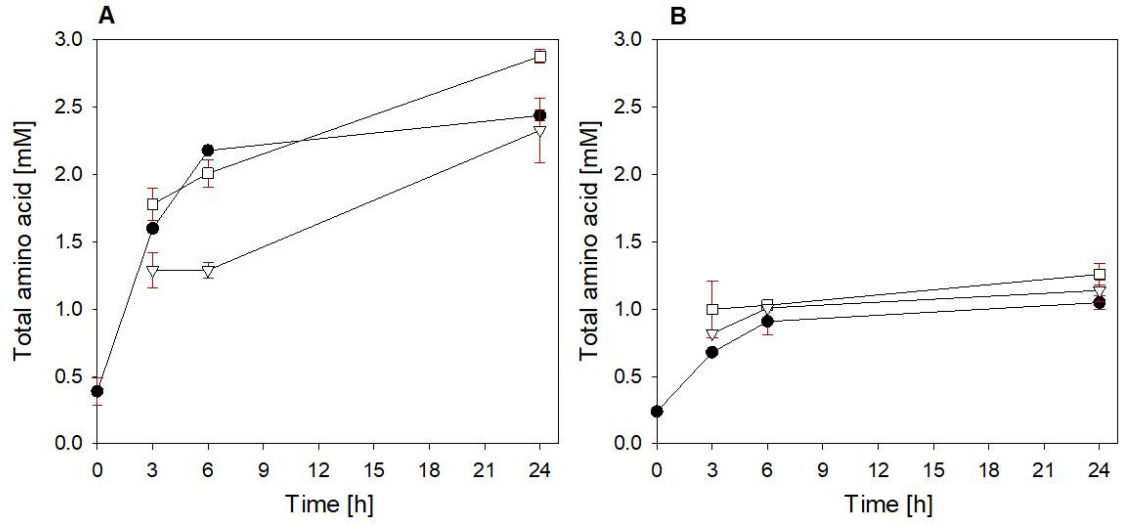


Figure 14: Analysis of 2nd enzymatic hydrolysis filtrates by OPA assay. Panel A: Lanreotide hydrolysed by Flavourzyme, panel B: Lanreotide hydrolysed by Pronase. Filled dots: amino acid content determined from filtrate of solely protease. Empty triangles: 0.2 mM Lanreotide hydrolysis, empty squares: 1.0 mM Lanreotide hydrolysis. Error bars are illustrated in red.

4.6 Purification of proteases

Three different purification methods were investigated, in either 5.0 mM PB, pH 7.0 or dH_2O. Protein content, amino acid content (determined spectrophotometrically on the basis of OPA-assay) and protease activity in each fraction, served to monitor protease purification. Additionally, the time required for each preparation was also taken into account to choose a suitable method. NAP-column purification took approximately 30 min, Vivaspin ultrafiltration procedure took approximately 2 h and dialysis took approximately 3 h in total. The aim of this study was to find an efficient method which was suitable for both proteases. Efficiency in this case was defined as a minimal loss of enzyme activity and protein content possible with a maximum reduction of autolysis amino acids. Table 18 gives an overview about studies conducted with both proteases applied and data acquired for each purification experiment.

Table 18: Purification of Flavourzyme and Pronase. "Initial" and "Purified" refers to values obtained before and after the purification method. Abbreviations: PC, protein content; AA, amino acid content; EA enzyme activity.

		Flavourzyme			Pronase		
Fraction	Volume [mL]	PC [mg]	AA [mmol]	EA [µmol/min]	PC [mg]	AA [mmol]	EA [µmol/min]
Vivaspin dH_2O							
Initial	0.50	4.36	38.24	13.66	0.33	1.73	3.56
Purified	0.10	1.97	3.40	2.79	0.19	0.15	1.50
Vivaspin 5.0 mM P, pH 7.0							
Initial	0.50	7.72	43.45	12.06	0.25	1.93	3.94
Purified	0.10	1.82	2.67	2.06	0.00	0.39	0.00
Dialyse dH2O							
Initial	0.10	4.26	37.52	10.12	0.32	1.82	2.74
Purified	0.28	3.14	4.39	1.57	0.27	0.57	3.26
Dialyse 5.0 mM PB, pH 7.0							
Initial	0.10	3.86	40.43	10.73	0.25	1.80	4.21
Purified	0.81	2.68	7.57	7.51	0.27	1.21	3.63
NAP dH2O							
Initial	0.50	3.41	38.01	11.78	0.29	2.04	3.02
Purified	1.00	4.41	12.15	9.09	0.16	1.20	0.88
NAP 5.0 mM PB, pH 7.0							
Initial	0.50	4.99	43.79	11.75	0.37	2.78	3.92
Purified	1.00	4.14	5.36	9.79	0.20	0.91	3.01

Figure 15 elucidates the effect of each purification method as a reduction in percent in bar charts (panel A: Flavourzyme; panel B: Pronase), referring to the initial values acquired prior to purification as 100%. By comparing initial to purified values of amino acid content in Table 18 (AA [mmol]), a significant decrease of roughly two thirds was perceived for all methods applied to Flavourzyme. Concerning Pronase, dialysis in buffer as well as NAP-column purification in dH_2O, reduced amino acid content only by a third. For both enzymes, Vivaspin ultrafiltration showed the largest reduction of amino acids, however, activity and protein content were decreased by more than 80% in buffer and between 40% and 60% in dH_2O.Taken the limitation of severe loss of activity into account, purification by Vivaspin ultrafiltration was not pursued further. Promising results for dialysis were noted for Flavourzyme in buffer with a significant reduction of amino acids and acceptable recovery of protein content and activity. On the contrary, reduction of amino acids of Pronase by dialysis in buffer was seen as insufficient with solely 32%.

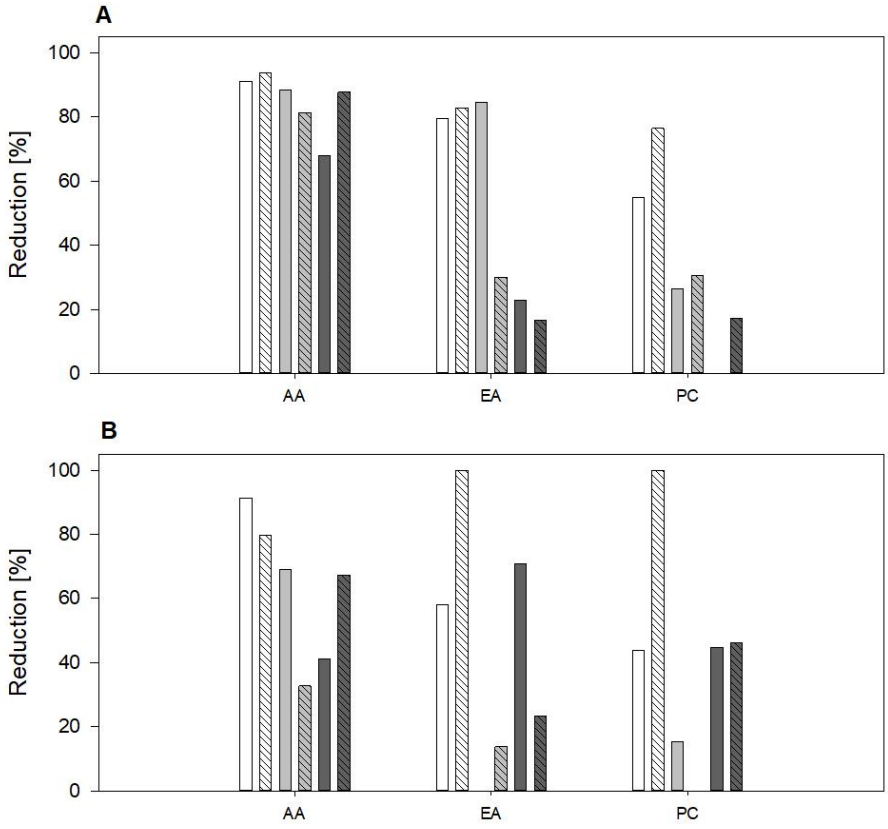


Figure 15: Effect of purification methods on Flavourzyme (panel A) and Pronase (panel B). Reduction [%] refers to initial values acquired before purification as 100%. White bars: Vivaspin ultrafiltration. Light grey bars: Dialysis. Dark grey bars: NAP-column purification. Bars with pattern indicate the usage of 5.0 mM PB, pH 7.0, while bars without pattern indicate the usage of dH_2O. Abbreviations: PC, protein content; AA, amino acid content. EA, enzyme activity.

NAP-column purification in buffer resulted in significant amino acid reduction of 95% and 67% for Flavourzyme and Pronase, respectively. Furthermore, the loss of activity constituted approximately a fifth from initial values for both proteases. Although the loss of protein for Pronase was in this case rather high with 46%, it was accepted as Pronase initially had approximately a fourfold higher specific activity than Flavourzyme.

It is known from literature that the two proteases applied, were actually a blend of endo- and exopeptidases. 10 different proteases are included in Pronase, whereas 8 proteases are found in Flavourzyme [85, 86]. An SDS-PAGE analysis was carried out to investigate whether or not a complete loss of one band, referring to a loss of 1 of the 8 or 10 proteases could be recognised after NAP or dialysis purification. A representation of the SDS-PAGE gel is displayed in Figure 16. It elucidates that the band patterns after purification were rather equal, but this could only be verified for Flavourzyme because for Pronase the amount of protein loaded was too low to clearly see differences. However, a complete loss of one band was not seen in this SDS-PAGE analysis for Flavourzyme. Based on results presented and as NAP-column purification can be conducted in less than 1 h, NAP-column purification using 5.0 mM PB, pH 7.0, was used for further protease purifications in this work.

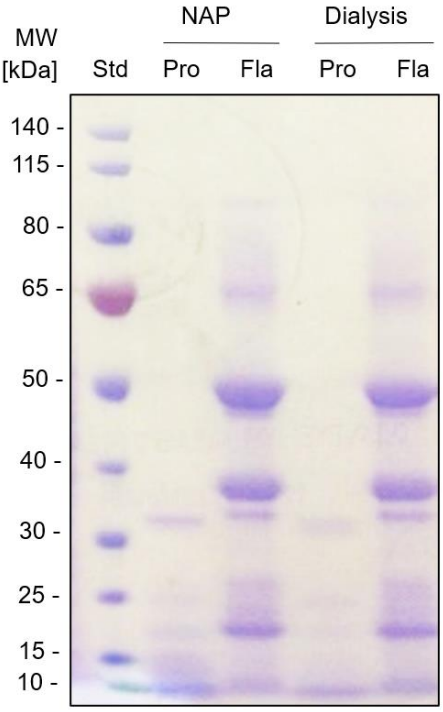


Figure 16: SDS-PAGE analysis of purified enzymes with either NAP-column or dialysis in buffer. Abbreviations: Std, Standard Page Ruler; Pro, Pronase; Fla, Flavourzyme.

4.7 Covalent immobilisation of proteases

Four studies were carried out in this work to immobilise proteases. In Study A three enzyme carriers were tested. In Study B the amount of immobilised protease used in the activity assay was evaluated. Furthermore, it should also be decided which of the two buffers used (700 mM/50 mM PB, pH 7.0) could be considered as superior. In Study C the degree of immobilisation was studied in dependence of the amount protease added to the solid carrier (100-1500 µg). Study D was carried out to investigate the stability of enzyme activity during immobilisation conditions without adding beads. Moisture content of beads was determined to account for dH_2O in Sepabeads EC-EP/S, which was necessary to calculate the exact amount of U/mg dry beads. A ratio ($r_{w/d}$) of 2.933 was determined for Sepabeads and used for further calculations.

Study A: Immobilisation with Chitosan did not show any binding of protease to the carrier. For Eupergit C a binding efficiency of 8.4% was determined for Pronase, while Flavourzyme showed no binding at all. The best results were obtained with Sepabeads, were approximately a sixth of enzyme offered bound to the carrier (Pronase 17.7%, Flavourzyme 14.8%). Subsequently, Sepabeads were used for further studies.

Study B: Flavourzyme produced more autolysis amino acids than Pronase as shown in Section 4.5.1. Furthermore, specific activity of Flavourzyme was fourfold lower compared to Pronase. Due to being the less active protease, and as only 14.8% of the protease bound to Sepabeads, measurement of immobilisate activity was thought to be problematic. Thus, further studies were conducted with Pronase.

700 mM PB, pH 7.0 was considered as advantageous because when 50 mM PB, pH 7.0 was used, no binding was observed, and with 700 mM PB, pH 7.0 roughly two thirds of protein amount loaded, bound to Sepabeads. Specific activity was different in either of the buffers: a specific activity of 13.42 U/mg and 2.93 U/mg was determined for Pronase before and after NAP-purification in 700 mM PB, pH7. In 50 mM PB, pH 7.0 18.31 U/mg and 13.86 U/mg were measured before and after NAP purification.

Although specific activity was significantly lower (by 77%) in 700 mM PB, pH 7.0, usage of this buffer obviously indicated a better binding behaviour for Pronase as two thirds of protein offered bound to the beads. Thus, 700 mM PB, pH 7.0 was further used as immobilisation buffer of choice. Absorbance values obtained from activity measurements with 10-40 mg wet beads applied in the assay were all considerably low (0.03 ± 0.01 [-]), thus calculated activites were similarly low either. An average activity of 0.25 ± 0.06 U/g_{dry} was determined for all bead

weights applied in the assay from 5-20 min incubation time. The lowest deviations of this value were found between 10 – 20 min, thus in subsequent activity measurement, initial rate of immobilisate was determined by measuring activity after 10, 15 and 20 min. A decrease of mixing efficiency was not noted by applying different amounts of beads in the assay, so usage of 10-50 mg can be recommended.

Study C: In contrast to Study A, only Pronase used and it was purified by gel filtration prior to usage. Specific activity after NAP purification accounted for 4.16 U/mg. Additionally, the bead amount used was doubled from 100 mg to 200 mg. By steadily increasing the amount of enzyme offered from 100 µg to 1500 µg per 200 mg beads in five steps, a limitation regarding enzyme loading was not observed (compare Figure 17, panel A). An increased immobilisation yield by 24% was determined, compared to study A. Enzyme bound per amount of protein loaded is shown in Figure 17, in percentage points (panel A). Instead of 17.73% (Flavourzyme) and 14.8% (Pronase) as reported in Study A, an average of 72.81 ± 8.63% of enzyme loaded, bound to the carrier (compare reference line Figure 17, panel A). Panel B indicates that beads activity measured was increasing in accordance with protein bound. However, Pronase was way less active than determined after NAP-purification.

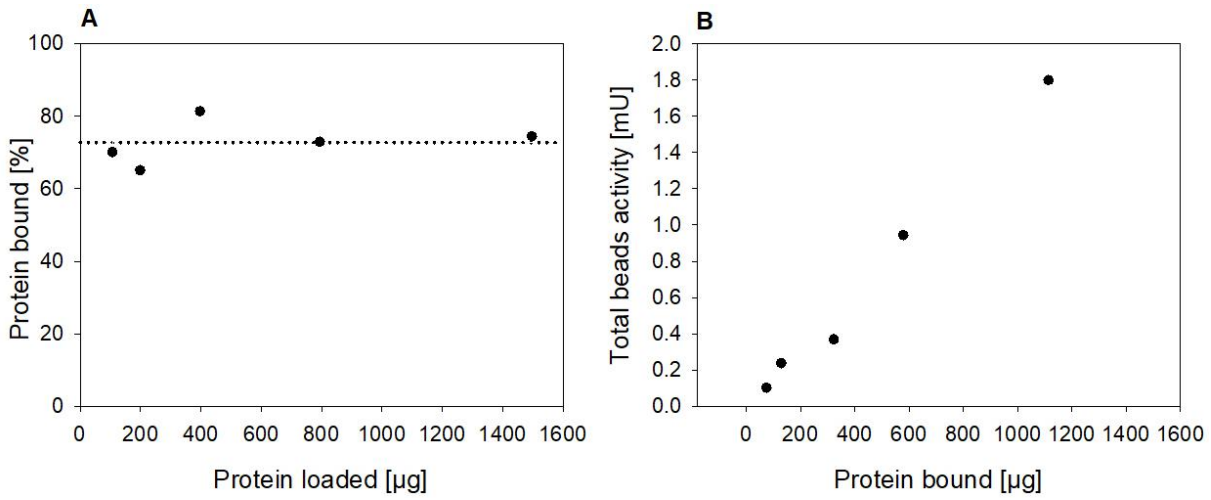


Figure 17: Study C - Immobilisation efficiency and activity recovery. Panel A indicates amount of protein bound in percentage points (100 – 1500 µg protein loaded). Reference line in panel A indicates the average binding efficiency of 72.81 ± 8.63%. Total activity [mU] of immobilisate is depicted in panel B. U refer to µmol L-Tyr released per minute in the casein assay.

Table 19 summarises expected and actual activities measured for supernatant and bead fractions in the study. For the highest amount of protein applied (1500 µg), a total immobilisate activity of 1.8 mU was determined. This value represented 0.029% of initially applied 6.23 U in total immobilisation reaction and 0.039% of the expected amount of U, calculated according to binding efficiency of 74% Table 19.

Table 19: Study C - Activity in supernatant and bead fractions. Expected values were calculated according to binding efficiency [%]. Actual values were obtained in the course of activity measurement. [%] given in supernatant and bead fractions refer to expected values as 100%.

Protein offered	Protein binding efficiency	Total U [µmol/min]	Supernatant U [µmol/min]			Beads U [µmol/min]		
[µg]	[%]	expected	expected	actual	[%]	expected	actual	[%]
100	70	0.445	0.133	0.064	48	0.312	$1.03*10^{-4}$	0.033
200	65	0.827	0.288	0.101	35	0.538	$2.38*10^{-4}$	0.044
400	81	1.654	0.309	0.215	70	1.345	$3.69*10^{-4}$	0.027
800	73	3.308	0.894	0.592	66	2.413	$9.43*10^{-4}$	0.039
1500	74	6.234	1.591	1.290	81	4.643	$1.80*10^{-3}$	0.039

Study D: Due to the extremely low activities measured in immobilisates of previous experiments, an activity decrease in a time-dependent incubation manner was a possible explanation. The exposure to room temperature for 24 h on the rotator were tested, thus no beads were used in otherwise identical conditions. 100 µg of Pronase were applied and a total activity of 0.54 U was determined in the solution at timepoint 0. After 24 h of incubation, an activity of 0.18 U was detected, which represented a loss of 67% of activity during incubation without beads.

Based on these findings the following conclusions can be drawn: Covalent immobilisation of 100 – 1500 µg Pronase is feasible with Sepabeads EC/EP-S. Pre-purifying the enzyme with a NAP column, and usage of 700 mM PB, pH 7.0 is recommended to successfully bind two thirds of protein offered on the carrier. Usage of 10-50 mg immobilised enzyme is recommended for carrying out the casein activity assay. Initial rate can be determined by stopping the reaction after 10, 15 and 20 min and calculating activity by applying linear regression. The extreme loss of activity during immobilisation, might derive from the time-dependent incubation over time.

5 Discussion

5.1 Photometric assays: adaptions made and improvements achieved

A broad variety of ready to use photometric assays for amino acid, protein or protease activity measurement is available in literature [101–102]. Though, these assays require adjustment to the infrastructure of the lab as for example down- or up-scaling of assay volume. Furthermore, calibration ranges need to be adapted to the desired measurement range. Moreover, incubation times have to be tested and if possible, shortened to increase efficiency. In this section, adaptions and improvements of photometric assays will be discussed.

5.1.1 OPA assay

Current mass spectrometric analysis methods have high operational costs and require time consuming data processing [104]. Consequently, monitoring the progress of peptide hydrolysis online in the lab is not possible. As an alternative we decided to test the OPA assay, with which a quick and rough estimation of degree of hydrolysis could be made, during the reaction takes place. It was attempted to, calibrate all amino acids present in each peptide. From the average molar extinction coefficient of each peptide, the content of free amino acids in hydrolysates could be estimated. Furthermore, it was tried to improve the preparation of the OPA. These points were taken into consideration to decide whether or not OPA assay is a suitable tool to monito hydrolysis efficiency.

Regarding calibration, a criticisable point of the OPA assay protocol from Interchim is the recommended calibration range of 0.8 - 12µM of α-acetyl-Lys [91]. This is a far too narrow range for calibration purposes in this study, where the full linear measurement range of the photometer was utilised. Interchim recommends – if necessary – to adapt the calibration range according to studies carried out. Therefore, the calibration range was extended to 0.05 – 2.0 mM, which could be applied to all measurable amino acids.

By calibrating all amino acids present in the peptide, only those amino acids that contained primary amines could be calibrated. This was in line with our expectation, because OPA does not build a complex with secondary amines. Thus, only 4 out of 9 amino acids in Icatibant could successfully be calibrated. For Lanreotide, this applied to all amino acids present in the peptide because all of them contained primary amines. Molar extinction coefficients were all within 0.36 – 0.58 mM^{-1}, except for L-Cys (0.27 mM^{-1}), so they were comparable. Subsequently an average molar extinction coefficient was calculated for each peptide (Lanreotide: ε = 0.48 ±

0.09 mL mM^{-1} (L-Cys excluded), Icatibant: $\varepsilon = 0.48 \pm 0.26$ mL mM^{-1}). According to Poole et al. [105] high pH values > 8 promote oxidation reactions. Two L-Cys could build Cystine, connected by a disulphide bridge. As a buffer of pH 10.5 was used for solving the OPA reagent, this oxidation could explain this decreased molar extinction coefficient of L-Cys. In preliminary studies (data not shown) OPA reagent was prepared in buffer at pH 8.0, 9.5 and 10.5. Absorbance values for L-Cys were on average a third lower, when OPA reagent at pH 10.5 was used compared to when OPA reagent at pH 8.0 was used. These findings would underline the pH dependency of Cystin formation described by Poole et al. [105].

Beside enlargement of the calibration range, usage of frozen stocks of OPA solutions was an improvement. By that, the assay was made more efficient and enabled the determination of amino acid content in samples withing 15 min. Compared to LC/MS measurements, which take several days from sample preparation to data analysis, this is a major advantage. To sum up, OPA assay allowed extending the calibration range, measuring primary amines reliably and the performance was by far less time consuming, than LC/MS measurements. Therefore, the assay can be recommended for amino acid analysis studies, as for example to analyse the degree of purification of a protease, done in this work.

Unfortunately, hydrolysis efficiency could not be estimated from results of OPA assay. Firstly, a high amount of hydrolysed amino acids were autolysis amino acids deriving from the proteases. Thus, the calculated degree of hydrolysis from OPA assay would be biased. For example, by hydrolysing 1.0 mM Lanreotide with Flavourzyme, OPA results indicated an amino acid content up to 2.8 mM. Secondly, from LC/MS data it was observed that hydrolysis efficiency deviated strongly from one to another amino acid (6-71%). To detect this deviation, estimate degree of autolysis and determine actual hydrolysis efficiency, LC/MS measurement is an indispensable prerequisite. Hence, OPA assay is a practical method to measure amino acid content, but it cannot be recommended to determine amino acid content for hydrolysates with a high amount of autolysis amino acids.

5.1.2 Bradford assay

Bradford assay was used to determine protein content of proteases. According to the manufacturer, buffer containing up to 100 mM (sodium) phosphate should not affect reactions with Roti®-Quant [106]. This is in line with our observations. However, for buffers containing 700 mM potassium phosphate, resulting calibrations differed strongly from those typically obtained. Slope values in this buffer were twice as high as the slope of standards prepared in dH_2O. Thus, for further works it is recommended to test potential interactions with buffers used for Bradford assay prior to measuring biological samples.

5.1.3 Protease activity assay

In the protease activity protocol provided by Sigma Aldrich several inconsistencies were noticed [103]. By carrying out the original protocol, phosphate and substrate concentration would vary throughout the assay. For example, Sigma Aldrich recommended to add different volumes of an enzyme solution (0 – 1 mL) in phosphate buffer, to a fixed volume of casein suspension (5 mL). By that, the end concentration of substrate and phosphate in the initial reaction was different (e.g., Casein: 5.4 – 6.5 g/L). This variation was eliminated by diluting the enzyme first and adding a constant volume of enzyme solution (100 µL) to a fixed volume of casein suspension (500 µL).

Beside this improvement, we could demonstrate that the reported concentration range of L-Tyr from 27.5 – 275.0 µM could be extended to 0.01 – 1.0 mM. In addition, the reduction of incubation times of casein digestion (from proposed 30 min to 6 min) and Folin reaction (30 min to 20 min) made the assay more efficient. Compared to the former protocol, the activity of a protease could be determined within 1 h instead of nearly 2 h. This assay is based on an unspecific digestion of casein, and therefore is a measure of total protease activity. Information about endo- and exo-specific activity cannot be gained. We further showed that the established protocol is suitable to determine activity of immobilised protease by using 10 – 40 mg immobilisate and applying multiple stopping times between 5 and 20 min.

5.2 LC/MS measurement and quality of LC/MS generated data

All LC/MS measurements were conducted at JOANNEUM RESEARCH HEALTH. Three runs were carried out in this work and samples obtained from hydrolysis were injected in a randomised order. Measurement inaccuracies were observed during the LC/MS measurement, possible reasons and implications of inaccuracies onto the data are discussed in the following. These include the injection mode (µL pickup/partial loop), the both methods (A, B) applied, peak integration, calibration, linear and phosphate trends, injection sequence and general recommendations for LC/MS measurement of amino acids.

5.2.1 LC/MS measurement setups and calibration

The first change in the measurement setup that affected quality of data from the 1st to the 2nd measurement was the change from µL-pickup method to a partial loop injection. The µL-pickup method was initially used because only a small sample volume (< 27 µL) is needed, and with the acquired filtrate more measurements of the same enzymatic hydrolysis could have been carried out. The major disadvantage of the µL-pickup method is that minor amounts of wash fluid are injected. Thus, the sample might be diluted and the peaks become less distinct. By switching to the partial loop injection, the needle tubing is flushed with sample before injection. 5 µL of sample are then discarded from the needle, which enables a clear sample front being loaded on the column. According to the manufacturers user manual, this method allows a more reliable reproducibility of measurement [107]. This could be verified in this study because dilutions better fit together, by choosing the partial loop injection mode.

Quality assessment of each measurement started with analysis of elution profiles. All amino acid peak signals which were found appeared within 10 seconds of the recommended retention times for Method A and B. Although the automatic integration modes where applied, a control of each peak is highly recommended, to manually correct peaks if necessary. The only measurement where peaks appeared fractured without clear borders was the 2nd, measured with Method B.

After integration of peaks, all compound-specific signals were transformed into molar units as described in Section 3.9.2. As explained, standard signals that did not fit the linear relationship between standard concentration and integrated area were excluded. In the 1st and 2nd measurement all standard signals obtained were used for calibration, however L-Ser and D-Arg (1st measurement, Method A) gave scattering peak signals which could not be used for a calibration. For the 2nd measurement, signals obtained from standards in the beginning were higher than those obtained in the end. This deviation will be discussed later in Section 5.2. During the 3rd

measurement, 5 out of 14 calibration points in several amino acids measured with Method A (L/D-Ser, D-Thi, D-Tic, Gly) had to be excluded (See Table 15). Of these, sometimes singular measurements in the beginning and sometimes in the end were affected. Causes for these rather random measurement failures yet remain unknown, but require further investigation in future works. Except for these measurement failures, acquired calibrations were all of acceptable quality with a correlation coefficient $R^2 > 0.90$, except for L/D-Cys in the 2nd measurement ($R^2 < 0.76$).

One further aspect of calibration was the linear measurable range. In the course of the 3rd measurement, the range was enlarged from 3.6 – 50 µM to 5 - 200 µM. The calibration points 1 and 2 (200 µM and 150 µM) had to be excluded in 10 and 7 out of 15 amino acid calibrations of Icatibant (see Table 15). This indicated a limitation for concentrations ≥ 150 µM, but the evaluation of the real upper limit of LC/MS amino acid quantification is reserved for further studies.

5.2.2 QCs and their role in trend analysis

After the calibration step, which was done directly on the LC/MS device, values obtained were analysed according to QC samples. These samples are used in mass spectrometry to detect systematic errors and to report the quality of measurements [108]. QCs were only measured once in the 1st measurement, which did not allow a quality assessment over time. Hence, they were measured multiple times during the 2nd and the 3rd measurement. A deviation of 15% of the expected values for QCs is usually accepted at JOANNEUM RESEARCH HEALTH. QC values in this work deviated by up to ± 40% (Compare Figure 4) which underlines the rather poor quality of measurements. By analysing QCs, linear trends over time were observed, which are discussed in the following.

Linear trends were noted in the 2nd and 3rd measurement, both measured with Method A. However, it affected once Lanreotide (2nd measurement) and once Icatibant (3rd measurement) amino acids. Besides QCs, standards which were measured throughout both measurements, were also used to assess the observed loss of signal. This loss of signal explains the previously stated deviation of the two calibrations in the beginning and in the end of the 2nd measurement. The trend correction was first applied to all standards as outlined in Section 3.9.2. By means of the correction, R^2 of calibrations was brought closer to 1 (compared to values before the correction). This indicates that the measured concentration is in accordance with the known concentration. Slope k was also brought closer to 1 and the standard error on k (Err_k) was lowered, which demonstrates that scattering of values was thereby reduced significantly. Thus, by applying the correction with regression parameters of each amino acid to all samples, more valid conclusions

about biological samples could be drawn. In literature, similar linear trends were also reported in previous studies [28].

Besides the linear trend correction, a phosphate correction was carried out. Previous studies reported that the presence of phosphate can impede the measurement [28, 29]. However, a correlation between increased phosphate concentration (up to 5 mM) and the necessity for a correction was not observed. In addition, the phosphate correction was only required for half of the amino acids of Lanreotide (see Table 14). Regarding the necessity of a correction, neither a trend towards L-, D- or artificial amino acids, nor towards polarity of affected amino acids was noted. It can be assumed, that stronger influences of phosphate are visible at higher phosphate concentrations than 5 mM.

The detection of these trends was only possible by applying QC samples and standards throughout the injection sequence. Adaptions of the initial sequence were made as outlined in Section 3.7.2 and findings indicate that the best evaluation of the quality of the measurement allowed the 3rd injection sequence (compare Section 3.7.2). An improvement would be to measure three instead of one QC throughout the measurement, after approximately each 10th sample measured. However, a disadvantage represents the increased time to measure one set of samples, when many QCs are applied, as each injection takes about 17 min. Another approach to assess the quality of measurements is internal standardisation. Therefore, stable isotope labelled amino acids are added to samples. Although internal standardisation has witnessed great interest in research [30, 109], isotope labelling is not performed on artificial amino acids yet, and is therefore not entirely applicable for this work.

5.2.3 Data analysis of biological samples

After integration of peaks, calibration and evaluation of trends, data of biological samples could be analysed. Dilutions made for each sample set deviated strongly in the 1st measurement and amounted up to 100% (see Table 11). These high deviations were reduced in the 2nd and 3rd measurement to about 30% deviation, due to measures taken like partial loop injection and corrections applied.

Furthermore, the calibration range of 3.6 – 50 µM and 5 – 200 µM was exceeded, regarding all L-amino acids found from enzymatic hydrolysis. Highest values were found for L-Lys and L-Thr (>2 mM), followed by L-Val (>1.6 mM). These high values can on the one hand be traced back to autolysis of the protease, and on the other hand to the exceedance of the calibration range. Accuracy of data is significantly lower outside the calibration range and the upper limit

of quantification, according to Zabell et al [110]. For biological samples this means that measured values above the upper limit of quantification are an underestimation of the actual values.

To conclude, the partial loop injection is recommended all LC/MS measurements, where sample volume is not limited. The applied Methods A and B were inconsistent regarding batch-to-batch performance. Singular measurement failures were observed during calibration, which is why many of the calibration points needed to be excluded during the 3rd measurement. This exclusion particularly affected standards of higher concentrations than 150 µM. Deviations of QCs underlined the poor quality of the measurement but indicated a time dependent linear trend, which was successfully corrected. Thus, application of QCs was proven to be crucial to asses quality of LC/MS measurements. An increasing phosphate concentration of up to 5 mM did not cohere with a significant deterioration of LC/MS signal. In addition, dilutions fit better in the 2nd and 3rd measurement, but in all cases the exceedance of the calibration range only allowed a rough estimation of data obtained from hydrolysis reactions. These findings underline the necessity of further research, regarding improvement of LC/MS measurements. Otherwise, current ICH qualifications of 0.5% impurity of the actual substance, cannot be fulfilled.

5.3 Comparison of enzymatic and acid peptide hydrolysis

Enzymatic and acidic hydrolysis of Lanreotide and Icatibant are in the following compared, regarding racemisation, degree of hydrolysis and amino acids that are either unstable or appeared at a high concentration. Furthermore, recommendations will be given for an enzymatic hydrolysis protocol, including parameters like temperature, phosphate and peptide concentration, incubation time and quality of enzyme. The more advantageous protease will be evaluated particularly based on hydrolysis efficiency. Lastly, potential cleavage patterns are analysed.

5.3.1 Racemisation is not introduced by enzymatic hydrolysis

Chiral amino acid stability was investigated in four different approaches. The effect of racemisation was investigated based on free amino acids during acidic or enzymatic hydrolysis conditions, or peptide bound amino acids hydrolysed by acid or enzymes. Both treatments of free amino acids, did either not produce chiral variants or they were found in extremely low concentrations or below the lower limit of quantification. It affected D-Thi and D-Ser and accounted on average for 1.5%, which could likely derive from outlined measurement inaccuracies, as these amino acids were measured with Method B during the 3rd LC/MS measurement set. Similar findings regarding acidic treatment of free amino acids where reported by Csapó et al. [36].

Peptide bound amino acids cleaved by acid hydrolysis showed racemisation of up to 9% and 14% for L-Nal and D-Cys. These values were obtained after 90 min of incubation and on

average, racemisation amounted 4% for Lanreotide and 1% for Icatibant. These results are in accordance with findings from Csapó et al. [36] and Kaiser and Brenner et al.[41] who reported a degree of racemisation of peptides during acid hydrolysis by 1-10%. Pellati et al. demonstrated that chiral inversions are both acid- and base-catalysed reactions that occurred predominantly at pH values between 1-3 and above 10 [111]. When hydrolysis is conducted for a longer time, amino acids bound in the peptide are longer exposed to a highly acidic surrounding which causes racemisation. As a result, factors which speed up hydrolysis, like the recommended short incubation time of 10 min, lower the degree of racemisation [36]. In general, the observed degree of racemisation is rather low. With respect to deviations seen during LC/MS measurements, these racemisation values constitute rather less of an issue than the measurement inaccuracy itself. In further studies, the degree of racemisation of many more peptides should be investigated. If a constant degree is noted in each batch, this value can be considered for quality control. Subsequently, despite racemisation, the correct composition of a peptide can be verified.

During enzymatic hydrolysis of peptides, chiral variants were not found above the lowest level of quantification and signals obtained rather reflect artefacts from LC/MS measurement, than proof for enzymatically introduced racemisation. Hence, it can be concluded that during synthesis of the peptide, solely the desired variant of each amino acid was incorporated in the structure. Incorrect enantiomers were therefore not undesired by-products of synthesis but induced by acid hydrolysis.

5.3.2 Despite drawbacks, acid outweighs enzymes

Given the fact that complete hydrolysis of the peptides could not be confirmed with neither of the methods, enzymatic and acidic approaches can still be contrasted. Both methods showed that degree of hydrolysis is affected with a high standard deviation between 14 and 28%. This deviation did not only derive from the measurement, but because the degree differed from amino acid to amino acid. For example, 4% of D-Tic, but 23% of L-Thi were cleaved from Icatibant. Moreover, it was observed that hydrolytic reactions take place rather fast, which also applied to both methods. Although the acid hydrolysis protocol of JOANNEUM RESEARCH HEALTH recommends an incubation time of 30 min (at 160 °C), it could be shown that 10 min are sufficient for both peptides, because a significant increase of degree of hydrolysis cannot be observed up to 90 min. Short reaction times of 15 min have also been advised by Westall et al. [35] and Csapó et al. [36] at similarly high degrees (160 °C) to accelerate the procedure and reduce racemisation events.

An average degree of acid hydrolysis of 61± 28% could be demonstrated in the present work, however L-Lys was mainly responsible for this high standard deviation. L-Lys had an average degree of hydrolysis of 108%, whereas for all other amino acids it was half as high. Hurrel et al. [112] reported that L-Lys was more stable during acid hydrolysis conditions than other amino acids like L-Val or L-Cys. The fact that 48% L-Val and 32% L-Cys, but 108% L-Lys were found in Lanreotide hydrolysates gives evidence for this assumption.

On the contrary, significant losses of L-Cys during acidic treatment of a protein were reported by Darragh et al. [113], which has also been demonstrated in this study for enzymatic as well as acid hydrolysis. Both, measurement as well as hydrolysis instability may account for the losses of L-Cys.

Considering stability studies, L-Oic was the only amino acid that was not found in the approximate amounts added in the AA-mix, but in peptide hydrolysis reactions it could be determined successfully. As for the preparation of free L-Oic an Fmoc-deprotection was required, an incomplete deprotection of Fmoc could explain this circumstance. Subsequently lower amounts of L-Oic are detected by LC/MS, as seen in Figure 9 (L-Oic 2 ± 0.3 µM).

Most significant enzymatic hydrolysis effects were seen within the first 6 h, with a subsequent stagnation, suggesting a biphasic reaction. The stagnation might be explained by a decrease of activity that coheres with steady autolysis reactions during the incubation at 50°C. More precisely, by cleaving conformational sites of proteases that retained their catalytic potential, activity is lost and hydrolytic reactions stagnate as shown in Section 4.5.2 and by Chen et al. [88]. This led to an approximate degree of hydrolysis of 20% with both enzymes applied, which could only be roughly estimated according to artificial and D-amino acids found. High amounts of autolysis amino acids hinder the exact determination of degree of hydrolysis.

The presence of enormous amounts of L-Lys in hydrolysates noted in this work, has already been reported by Maneepun et al. [114]. They proposed that a microbial protease from *Streptomyces* favours cleavage sites adjacent to positively charged amino acids like L-Lys. L-Lys residues in an exterior position of the proteases could according to this rationale be cleaved more often than other amino acids. Although their study did not directly investigate proteases of Flavourzyme and Pronase, it is a possible scenario, why more L-Lys was cleaved from proteases, compared to all other amino acids.

5.3.3 Enzymatic hydrolysis

Parameters of interest during enzymatic hydrolysis reactions were the time of incubation, phosphate concentration, the amount of peptide added and the quality of enzyme added (purified, non-purified). A detailed description of the applied protocol can be found in Section 3.5.1.

As outlined in the previous section, an incubation time of not more than 6 h is recommended, mainly because 90% of the peptide hydrolysis takes place during this time. Specific enzyme activity was similar in 5 mM as well as in 50 mM PB, pH 7.0, though, a rather low phosphate concentration is recommended, as increased salt concentration impedes the LC/MS measurement, demonstrated in previous studies [28, 29]. Higher substrate concentrations (1.0 mM) are superior to low substrate concentrations (0.2 mM). Basically, because when autolysis L-amino acids were subtracted from the complete hydrolysis results, results from 0.2 mM samples could not be distinguished from standard errors. Furthermore, it was assumed that if more substrate is available, proteases would rather tend to cleave the peptide instead of themselves.

Concerning enzyme quality, it was demonstrated that purification of proteases is essential prior to utilizing proteases in peptide conversions. The tested amount of enzyme was 10 U. This amount was suitable to detect the hydrolysis reaction, but produced high amounts of autolysis amino acids. It remains to clarify, whether lower amounts of enzyme could still conduct hydrolytic reactions with a similar efficiency but do not mask the actual degree of hydrolysis due to autolysis amino acids.

The chosen temperature of 50°C represented the temperature of optimal activity for Flavourzyme. A reduction of this temperature could be a thinkable measure to limit autolysis processes, as outlined by Chen et al. [115]. Furthermore, a reduced reaction temperature would be favourable for Pronase, as this protease has its activity maximum between 35 – 40°C, as stated by Nomoto et al. [86].

Incorporation of D- and artificial amino acids limited undesired degradation of therapeutic peptides by proteases, as reported by Marqus et al. [8]. Time-course experiments with Lanreotide showed this phenomenon exemplarily by D-Trp. In comparison to all other amino acids, where on average 90% of cleavage happened within the first 6 h, D-Trp was released steadily, during 24 h of incubation. After 24 h, Flavourzyme released 20% of the available D-Trp of Lanreotide.

The subtraction of autolysis amino acids from hydrolysis of 1.0 mM Lanreotide, in this work, demonstrated that the amount of L-amino acids liberated was on average the double amount of the portion of D-amino acids detected, which coincides with findings from literature. According

to Miller et al. [116] and Carmona et al. [117], D-amino acids are cleaved less effective than L-amino acids. A suchlike differentiation was exclusively observed for enzymatic and not for acidic hydrolysis, which underlines another benefit for acid hydrolysis.

With the outlined recommendations, a degree of enzymatic hydrolysis of 44 ± 27% was evaluated for hydrolysing 1.0 mM Lanreotide with Pronase, and a degree of 20 ± 14% was demonstrated for Flavourzyme. Due to the fact that these two values are statistically not clearly distinguishable, the advantages and disadvantages of either of the proteases are outlined in the following.

5.3.4 Evaluation of advantageous protease candidate

In regard of enzyme preference, Pronase was superior to Flavourzyme for two reasons. Firstly, Pronase produced on average a third less autolysis amino acids. Secondly, specific activity was four times higher than specific activity of Flavourzyme, which could give rise to faster enzymatic hydrolysis reactions, if conducted at the optimal activity temperature (35-40 °C) for Pronase.

A distinction of proteases applied, according to artificial and D-amino acids cleaved of peptides cannot be made, because both enzymes cleaved at best 20% of these amino acids. Namely, Pronase cleaved more D-Trp and Flavourzyme cleaved more D-Nal. Yet, the subtraction of autolysis amino acids from time-course experiments with Lanreotide showed, that the average degree of hydrolysis (44 ± 27%) was approximately twice as high for Pronase than for Flavourzyme (20 ± 14%). By excluding L-Lys from the calculation, the values are 32 ± 8% for Pronase and 17 ± 14% for Flavourzyme. In addition, the relative standard errors are thereby lower, especially for Pronase (0.25 instead of 0.61).

D-Tic and L-Thi are positioned in the midst of Icatibant, and D-Nal represents the carboxyl terminus of Lanreotide. All of these amino acids were cleaved by both enzymes which indicates that both enzymes are capable of breaking peptide bonds from the inside and from the outside, regardless of the type of amino acid (L-, D-, artificial). This gives evidence for endo- and exoprotease activies in both enzymes. Yet, in all enzymatic hydrolysis reactions Pronase cleaved amino acids more efficiently and conducted less autolysis which speaks for this protease.

5.3.5 Cleavage analysis: Theoretical Lanreotide hydrolysis by a protease cocktail

Delatour et al. reached an enzymatic degree of hydrolysis of 75% by using Pronase and Aminopeptidase in combination to hydrolyse BSA [73]. The study demonstrated that either of the proteases cleaves some amino acids more efficiently than the other protease. For example, D-

Trp, L-Tyr, L-Thr and L-Lys, was liberated to a higher proportion by Pronase, whereas more D-Nal and L-Val has been produced by Flavourzyme. Theoretically, by using Flavourzyme and Pronase in a suchlike one-pot reaction, Lanreotide could be cleaved completely but to a higher extent. Figure 18 illustrates this theoretic full-digest by both enzymes, where all amino acids are set free, except for the two L-Cys (Cystin) which are still connected by the disulphide bridge.

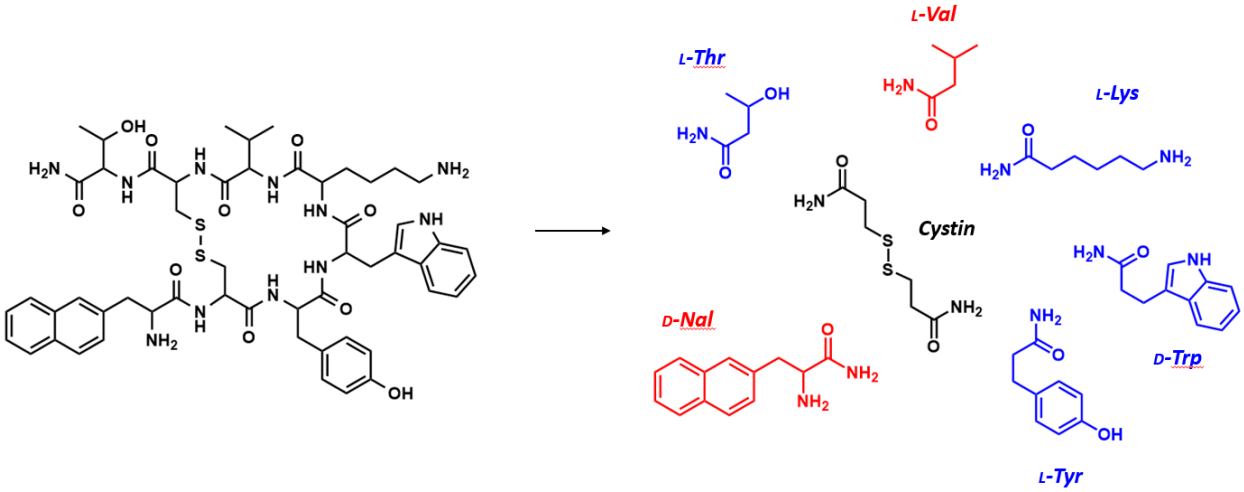


Figure 18: Structural formular of an intact (left) and hydrolysed (right) Lanreotide molecule. Red amino acids are cleaved more efficiently by Pronase, blue amino acids are cleaved more efficiently by Flavourzyme. Two L-Cys build Cystin, connected by the disulphide bridge which was not cleaved by any of the proteases.

Capiralla et al. [118] and Hersh et al. [119] demonstrated in a comparative study of endopeptidases of different sources (*Halobacterium halobium, Bacillus thermoproteolyticus* and rat kidney) that endopeptidases had a substrate specificity for hydrophobic amino acids. Flavourzyme contains three endopeptidases and in the present work it was shown that the highest amounts of hydrolysed amino acids were L-Tyr, L-Val and D-Nal (which is originally an aromatic derivative of L-Ala). These amino acids are all hydrophobic, so a cleavage preference for hydrophobic amino acids is in good agreement with literature.

For Pronase a chemical specificity based on the composition of the mixture could not be found. Although Pronase consisted of 7 aminopeptidases (2 with L-Leu and 5 with L-Ser specificity), the most cleaved amino acid of Lanreotide was not its N-terminal end L-Thr, but L-Lys which is centrally located in the peptide.

For Icatibant, a similar digestion analysis could not be carried out, as values obtained from LC/MS data were too erroneous to allow quantitative interpretations.

5.4 Strategies to reduce autolysis

The high amount of L-amino acids, observed during all enzymatic conversions could be traced back to being products of autolysis of the protease. This effect has also been observed and reported by others. [114, 115, 120–122]. To reduce autolysis, different approaches have been investigated. For example, Kapust et al. [122] and Mildner et al. [123] proposed mutagenesis of autolysis sites by amino acid substitution. Maneepun et al. recommended acylation with dicarboxylic acid anhydrides to chemically modify all NH_2 groups to negatively charged carboxylic groups. This electrostatic repulsion can prevented a self-digest of proteases [114]. These methodologies are interesting and of course relevant for future enzymatic hydrolysis studies, but as they are rather elaborate and time-consuming, they extended the scope of the present study. In this work, purification and immobilisation of proteases was investigated to reduce autolytic effects and are discussed in the following.

5.4.1 Purification of proteases

Three different purification methods were tested, amongst which the most significant reduction of free amino acids surrounding the protease was achieved by NAP-purification. The purification amounted 95% and 67% for Flavourzyme and Pronase, respectively. Yet, Barach et al. [124] and Chen et al. [88] indicated a linear relationship between the increase of temperature and increase of activity which entailed an increase of autolysis. Reduction of autolysis by NAP column purification conducted on ice could reduce the amount of free L-amino acids significantly, however by using these proteases at 50°C, autolytic reactions increase and mask the actual hydrolysis of peptide related L-amino acids.

Besides the analysis of loss of amino acids and maintenance of enzyme activity, the time required for each preparation was also considered. It was attempted to limit the setup of an enzymatic hydrolysis experiment to one full day of lab work, to reduce influencing factors like variations of temperature, longer storage conditions or potential activity losses over time. As the setup of one experiment included determination of specific activity (1 h), purification of proteases (30 min), and preparation of substrate and the hydrolysis itself (1 h), the protocol of NAP-column purification was seen as advantageous to dialysis (3 h) or ultrafiltration (2 h).

In this study, the achieved purification was also evaluated by SDS-PAGE. Instead of clear bands for each protease in the mixture, additional bands were displayed in the gel (see Section 4.6) which could derive from autolysis. A recent study conducted by Fujita et al. in 2019 reported that although SDS was added to the sample to denature the protease, prior to loading the gel, the proteases were still active and conducted autolysis during the electrophoresis process.

Addition of 0.05 M sulphuric acid prior to SDS-PAGE reduced self-digest and allowed an improved purity determination of proteases of interest [100]. This approach could also be interesting for further SDS-PAGE analysis of proteases.

5.4.2 Immobilisation of proteases

Immobilised enzymes allow the easy recovery of enzyme and product and multiple-use of enzyme [79]. Furthermore, since immobilisation separates proteases locally from each other on a polymer, it prevents them from using neighbouring proteases as substrate. In other words, immobilisation limits autolysis and as numerous studies have been devoted to this theorem [80, 82, 114, 125], it was viewed as a promising method applicable in this work.

Successful immobilisation of proteases on the supports Eupergit C and Chitosan were reported by others [81, 98]. Unfortunately, in the present study, these carriers did either show unsuitable protein binding or completely differing values for both enzymes applied (0% Flavourzyme, 22% Pronase). Therefore, the third carrier, Sepabeads EC/EP-S was used for further investigation.

Basically, NAP-column purified Pronase immobilised on Sepabeads EC/EP-S was regarded as the most promising method, due to acceptable protein binding yields of around two thirds. The protocol included solely adding enzyme, and carrier in a 700 mM, pH 7.0 buffered solution [126]. This was an advantage to protocols found in literature that recommended quite demanding pre-treatment of carrier [81, 127]. The utilised buffer indicated sensitivity of Pronase activity to buffer concentration, because when 50 mM PB, pH 7.0 was used for the purification a minor loss of activity by 30% compared to activity before the purification was detected. However, when 700 mM PB, pH 7.0 was used a loss of activity by 80% was recognised. Still, protein binding was more efficient by using the buffer with the higher potassium phosphate concentration.

Unfortunately, immobilised proteases showed only a very low activity compared to the expected one (compare Table 19). There is likelihood that a temperature-related structural deformation could account for this considerable decrease of activity [128]. In general, the immobilisation was performed at room temperature and a 24 h incubation of Pronase on the circular rotator without beads showed a similar loss of activity (by 67%). Thus, incubation at room temperature was regarded as the major contributor to activity loss, in contrast to shear forces.

Further data collection would be required to determine how salt content and immobilisation affects protease activity. Moreover, different supports like alginate [82, 129] or magnetic nanoparticles [130] were found in literature and a complete recovery of protease activity was reported in these studies. In addition, Sarathi et al. recently reported increased temperature, pH, storage and organic solvent stability of immobilised keratinolytic protease on nanofibers [125]. These findings indicate that with the correct carrier and a suitable protocol, proteases like Pronase and Flavourzyme could also be successfully immobilised.

To conclude, immobilisation of Pronase and Flavourzyme on Sepabeads EC/EP-S was not regarded as a suitable tool up to this point to reduce autolysis, and conduct peptide hydrolysis with an immobilised protease, basically because activity yields of the immobilisate were extremely low.

6 Conclusion

In this Master's Thesis two protease mixtures, Pronase and Flavourzyme were used to evaluate feasibility of enzymatic hydrolysis of the pharmaceutical peptides Lanreotide and Icatibant, in comparison to currently applied acid-based hydrolysis. In order to monitor enzymatic hydrolysis reactions online and determine protease activity, photometric methods were adapted, partly improved and utilised in the present study. By the applied enzymatic hydrolysis protocol, a degree of enzymatic hydrolysis by at least 20% was achieved by using either of the proteases. This value was obtained by considering artificial and D-amino acids cleaved by the proteases, because in both enzymes a considerably high portion of L-amino acids deriving from autolysis was observed. Based on photometric as well as mass spectrometric measurements it was demonstrated that the portion of autolysis amino acids could be reduced by NAP-column purification. Further reduction of autolytic reactions was hoped to be achieved by immobilisation, however due to the severe loss of protease activity, the applied immobilisation method was not regarded as a suitable tool for enzymatic hydrolysis.

The validity of results was limited by the rather poor quality of LC/MS measurements. Nevertheless, measuring QC samples and calibration points multiple times and applying linear trend and phosphate correction to a complete measurement set cushioned the observed data impeding effects.

By definition, enzymatic hydrolysis of therapeutic peptides is feasible, but in comparison to acid hydrolysis, it was not considered as advantageous. Despite the fact that a slight degree of racemisation of several D- and artificial amino acids was observed, the correct composition of a pharmaceutical drug can still be verified more reliably by acid than by enzymatic hydrolysis. Further experimental investigations should firstly aim at enhancing quality of batch-to-batch LC/MS measurements and secondly, focus on the development of more stable acid hydrolysis techniques. Overcoming these bottlenecks is essential to meet future quality guidelines of the pharmaceutical industry.

7 Appendix

Table 20: Quality of amino acid calibrations of the 2nd LC/MS measurement. Linear range refers to range of concentration from which absorbance values were used to apply linear regression. Abbreviations: AA, amino acid; R^2 correlation coefficient.

Lanreotide AA	LC/MS Method	R^2	Linear range [µM]
L(D)-Thr	A	0.905	3 – 17
L-Val	A	0.901	10 – 48
D-Val	A	0.918	3 – 17
L-Trp	A	0.970	3 – 17
D-Trp	A	0.940	10 – 50
L-Lys	A	0.967	10 – 48
D-Lys	A	0.957	3 – 15
L-Tyr	A	0.901	10 – 50
D-Tyr	A	0.939	3 – 17
L-Nal	A	0.931	3 – 15
D-Nal	A	0.947	10 – 49
L-Cys	A	0.751	10 – 50
D-Cys	A	0.755	3 – 15

Table 21: Quality of amino acid calibration of the 3rd LC/MS measurement. Linear range refers to range of concentration from which absorbance values were used to apply linear regression. Abbreviations: AA, amino acid; R^2 correlation coefficient.

Lanreotide AA	LC/MS Method	R^2	Linear range [µM]	Icatibant AA	LC/MS Method	Linear range [µM]	R^2
L-Thr	A	0.981	5-200	L-Pro	B	5-100	0.985
D-Thr	A	0.994	5-200	D-Pro	B	5-200	0.964
L-Val	A	0.990	5-200	D-Hyp	B	5-200	0.973
D-Val	A	0.995	5-200	L-Hyp	B	5-200	0.974
L-Trp	A	0.986	5-200	L-Oic	B	5.200	0.970
D-Trp	A	0.996	5-200	D-Oic	B	5-200	0.957
L-Lys	A	0.999	5-200	L-Ser	A	5-200	0.991
D-Lys	A	0.995	5-200	D-Ser	A	5-150	0.931
L-Tyr	A	0.991	5-200	L-Thi	A	5-200	0.962
D-Tyr	A	0.997	5-200	D-Thi	A	5-150	0.936
L-Nal	A	0.993	5-200	L-Tic	A	5-200	0.968
D-Nal	A	0.998	5-200	D-Tic	A	5-150	0.917
L-Cys	A	0.969	5-200	L-Arg	A	5-200	0.958
D-Cys	A	0.974	5-200	D-Arg	A	5-150	0.948
				Gly	A	5-200	0.963

Table 22: Regression parameters of Icatibant standards measured with Method A during the 3rd measurement, before and after correction. $\frac{c_m}{c_k}$ refers to measured *(c_m)* and known *(c_k)* concentrations. $\frac{c_{corr}}{c_k}$ refers to corrected standard values (c_{corr}) plotted over known (c_k) standard concentrations. Abbreviations: Err_k, error of slope *k*. R^2 correlation coefficient; Ica, Icatibant.

Ica	Before linear trend correction				After linear trend correction			
AA	$k\frac{c_m}{c_k}$ [µM]	Err_k	$d\frac{c_m}{c_k}$ [µM]	R^2	$k\frac{c_{corr}}{c_k}$ [µM]	Err_k	$d\frac{c_{corr}}{c_k}$ [µM]	R^2
L-Pro	0.95 ± 0.08		1.11	0.929	0.98 ± 0.04		0.87	0.983
D-Pro	1.02 ± 0.07		-0.23	0.948	1.08 ± 0.08		0.12	0.938
D-Hyp	0.75 ± 0.07		0.85	0.897	0.87 ± 0.03		0.45	0.986
L-Hyp	1.24 ± 0.09		-0.44	0.943	1.08 ± 0.05		0.12	0.976
L-Oic	0.79 ± 0.05		1.04	0.954	0.74 ± 0.03		0.52	0.983
D-Oic	0.98 ± 0.07		-0.13	0.944	0.98 ± 0.04		-0.29	0.975

Table 23: Complete dataset of the 2nd enzymatic hydrolysis. Standard error was obtained from duplicates.

	Amino acid	Protease				Lanreotid hydrolysis 0.2 mM			Lanreotid hydrolysis 1.0 mM		
		0 h	3 h	6 h	24 h	3 h	6 h	24 h	3 h	6 h	24 h
Flavourzyme	L(D)-Thr	271 ± 29	989 ± 210	942 ± 262	1296 ± 377	739 ± 403	873 ± 464	1121 ± 254	1086 ± 172	1083 ± 174	1686 ± 349
	L-Val	228 ± 41	732 ± 103	785 ± 212	992 ± 313	412 ± 297	665 ± 347	824 ± 167	614 ± 102	595 ± 115	1083 ± 203
	D-Val	-	-	-	-	-	-	-	-	-	-
	L-Trp	32 ± 21	245 ± 42	199 ± 45	312 ± 56	232 ± 181	239 ± 191	325 ± 52	173 ± 23	175 ± 19	295 ± 43
	D-Trp	-	-	-	-	-	-	41 ± 2	-	9 ± 3	121 ± 10
	L-Lys	536 ± 439	1877 ± 105	1635 ± 264	2345 ± 306	1563 ± 575	1910 ± 1550	2553 ± 434	1235 ± 141	1200 ± 71	2311 ± 228
	D-Lys	-	-	-	-	-	-	-	-	-	-
	L-Tyr	73 ± 15	612 ± 139	575 ± 163	800 ± 233	131 ± 440	364 ± 440	407 ± 470	471 ± 59	492 ± 59	936 ± 170
	D-Tyr	-	-	-	-	-	-	-	-	-	-
	L-Nal	-	-	-	-	3 ± 0	5 ± 3	4 ± 0	-	-	-
	D-Nal	-	-	-	-	8 ± 14	10 ± 12	16 ± 19	54 ± 7	76 ± 8	193 ± 38
	L-Cys	-	15 ± 0	15 ± 0	23 ± 9	12 ± 0	9 ± 4	17 ± 0	20 ± 5	14 ± 0	18 ± 2
	D-Cys	-	-	-	-	-	-	-	-	-	-
Pronase	L(D)-Thr	264 ± 64	922 ± 175	855 ± 189	1074 ± 305	761 ± 124	777 ± 160	895 ± 187	477 ± 593	1444 ± 807	1329 ± 217
	L-Val	260 ± 100	663 ± 83	598 ± 161	700 ± 190	354 ± 409	681 ± 394	704 ± 408	886 ± 0	1096 ± 635	946 ± 164
	D-Val	-	-	-	-	-	-	-	-	-	-
	L-Trp	33 ± 22	212 ± 29	190 ± 5	232 ± 58	202 ± 24	168 ± 18	184 ± 5	169 ± 0	181 ± 111	166 ± 26
	D-Trp	-	-	-	-	16 ± 3	27 ± 1	71 ± 6	-	-	206 ± 22
	L-Lys	510 ± 401	639 ± 71	634 ± 49	770 ± 127	824 ± 62	792 ± 36	955 ± 56	454 ± 532	1096 ± 829	1186 ± 63
	D-Lys	-	-	-	-	1 ± 2	-	3 ± 4	-	-	-
	L-Tyr	69 ± 13	464 ± 101	430 ± 80	504 ± 134	242 ± 4	505 ± 4	554 ± 16	280 ± 459	1105 ± 459	880 ± 38
	D-Tyr	-	-	-	-	-	-	-	-	-	-
	L-Nal	-	-	-	-	-	-	-	-	-	-
	D-Nal	-	-	-	-	2 ± 1	3 ± 2	5 ± 3	-	49 ± 28	69 ± 16
	L-Cys	-	-	-	-	8 ± 2	9 ± 0	10 ± 2	-	-	-
	D-Cys	-	-	-	-	-	-	-	-	-	-

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