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Isolation, purification and investigation of peptides from fish proteins with blood pressure decreasing properties

Margrét Geirsdóttir

Líftækni og lífefni

Skýrsla Matís 36-09
Desember 2009

ISSN 1670-7192

Skýrsluágrip Matís ohf
Matis Food Research, Innovation & Safety
Report Summary

ISSN: 1670-7192

Titill / Title	**Isolation, purification and investigation of peptides from fish proteins with blood pressure decreasing properties** / Einangrun, hreinsun og rannsóknir á blóðþrýstings-lækkandi peptíðum úr fiskpróteinum		
Höfundar / Authors	*Margrét Geirsdóttir*		
Skýrsla / Report no.	36-09	*Útgáfudagur / Date:*	Desember 2009
Verknr. / project no.	0416 1787		
Styrktaraðilar / funding:	*AVS rannsóknasjóður í sjávarútvegi*		
Úrdráttur á íslensku:	Markmið verkefnisins var að rannsaka virkni í fiskpeptíðum og einangra, hreinsa og skilgreina peptíð sem hafa blóðþrýstingslækkandi áhrif. Í verkefninu var sett upp aðstaða og þekkingar aflað til þessa hjá Matis. Þar með er talin aðferð til að mæla ACE hindravirkni ásamt búnaði til einangrunar og hreinsunar á peptíðum. Í samstarfi við Háskóla Ísland var HPLC og Maldi-Tof búnaður nýttur til að greina hvaða peptíð voru í hinum virku þáttum. Niðurstöður verkefnisins sýna að íslensk fiskprótein gætu verið mikilvæg uppspretta peptíða með blóðþrýstingslækkandi eiginleika. Með þeirri þekkingu og aðstöðu sem hefur verið aflað í verkefninu er hægt að þróa verðmætar fiskafurðir og heilsufæði.		
Lykilorð á íslensku:	*Ensímniðurbrot, peptíð, lífvirkni, ACE, einangrun*		
Summary in English:	The aim of this project was to study the activity of fish proteins and isolate, clarify and define peptides with antihypertensive properties. During the project time methods and equipment to be able to do this were set up at Matis facilities. This includes method to measure ACE inhibition activity as well as filtration and fractionation units to isolate different fractions of peptides. Furthermore peptides have been identified in the most active fraction by using HPLC and Maldi-ToF equipment in collaboration with the University of Iceland. With this extensive tool box of knowhow, equipment and facilities, development of valuable fish products and nutraceuticals from blood pressure-lowering peptides is possible. Thereby the value of the Icelandic natural resources in the sea can be increased.		
English keywords:	*Hydrolysis, peptides, Bioactive properties, ACE, fractionation*		

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1. INTRODUCTION

1.1 Background

Proteins are important in food production both for their nutritional properties but also due to their functional properties that influence quality and consumer acceptance of food (Kristinsson and Rasco, 2000). Furthermore, peptides that are inactive within proteins have showed physiological effects in the body and when released shown function as regulatory compounds with hormone-like activity (Vermeirssen and others 2004; Hartmann and Meisel 2007). Those peptides have been named bioactive peptides (Birgisdottir, 2002; Tanaka and others, 2006). Peptides have also been investigated for their sensorial properties and can have a noteworthy effect on sweet and bitter taste in food products (Careri and Mangia, 2003). Functional properties of proteins can be modified by different means. The method which is most widely used is hydrolysis by proteolytic enzymes (Kristinsson and Rasco, 2000). Currently the most commonly used proteins in the food industry originate from soybeans or milk where sale has increased considerably in recent years (Jansen and Krijger, 2003). Different biological properties have been attributed to peptides and they have been shown to have influence as antioxidants, antimicrobials, surfactant agents and angiotensin converting enzyme (ACE) inhibitory activity (Murray and FitzGerald, 2007).

ACE is a multifunctional enzyme that plays an important physiological role in the regulation of blood pressure (Skeggs and others, 1956). Coronary heart disease (CHD) is one of the primary causes of premature death in many of the western world countries where increased blood pressure is one of the main risk factors. It has been estimated that by 2020 heart disease and stroke will become the leading cause of death and disability worldwide (WHO, 2009). In a big research named The OmniHeart (Optimal Macro-Nutrient Intake to Prevent Heart Disease) trial it was demonstrated that partial substitution of carbohydrate with protein sources low in saturated fat can lower blood pressure, improve lipid levels, facilitate short-term weight loss and reduce the risk of CHD (Appel and others, 2005). The mechanisms by which protein could exert its beneficial effects include an increased intake of biologically active amino acids or

peptides (Appel, 2003). In those researches the main focus has been on proteins of plant origin like soy.

These and other findings have increased the awareness of the critical link between diet and health. Moreover, they have led to the development of nutritionally enhanced food products designed to suit specific health concerns, particularly with relevance to the management of lifestyle-related diseases (Erdmann and others, 2008). Such foods, termed functional foods or nutraceuticals, are generally defined as products that have been satisfactorily demonstrated to have positive effects on one or more functions in the body, beyond their nutritional properties, in a way which is relevant to either an improved state of health and well-being and/or reductions of disease risk (Erdmann and others, 2008). The market for functional foods is big and is growing (Figure 1).

In Japan (Calpis) and Finland (Evolus) fermented milk products containing bioactive peptides are found on the market and can according to research lower blood pressure with regular consumption (Mäyrä-Mäkinen, 2003).

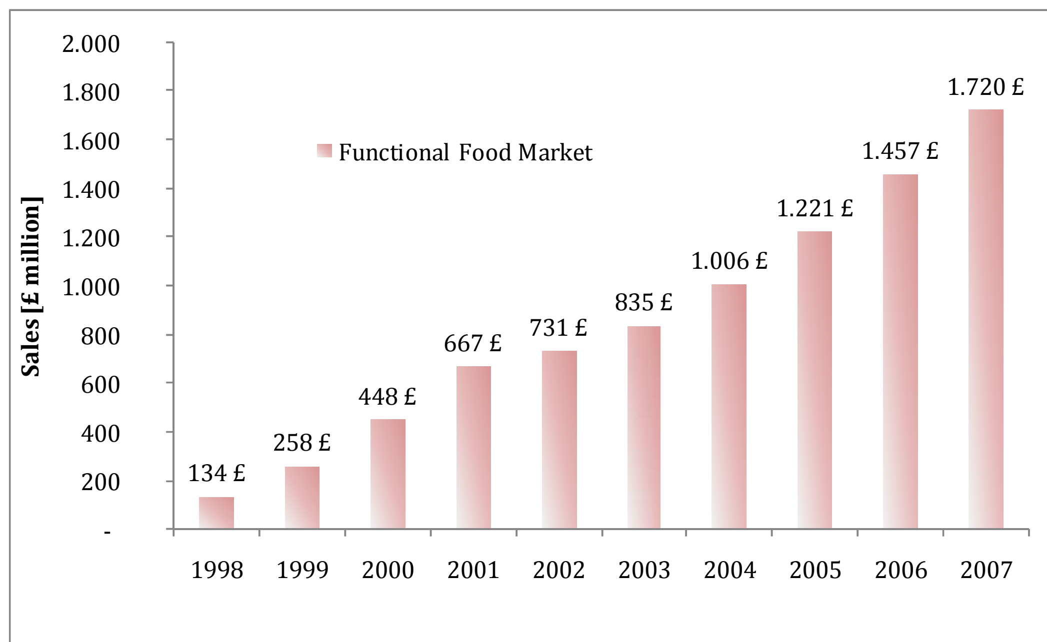

Figure 1. UK Functional Food and Beverage Products 1998 to 2007 (IGD, 2009).

Bioactive properties have mainly been screened by using *in vitro* tests. The drawback on those measurements is that only peptides containing a few amino acid residues are able to cross the digestive epithelial barrier and reach the blood vessels. It's evident that to be able to have beneficial effects for the organism the peptide must reach the peripheral organs (Yust and others, 2003). Direct connection between ACE inhibitor activity detected *in vitro* and anti-hypertensive activity *in vivo* is therefore hard to determine because of different bioavailability of peptides (Foltz and others, 2007). Furthermore peptides are prone to digestion during gastrointestinal passage and absorption (Foltz and others, 2009). Natural peptides lack metabolic stability and only a minority of the ACE inhibiting peptides will reach their site of action (Vermeirssen and others 2004).

Those are not the only obstacles when a new product of peptides derived from food proteins are to be developed. Other obstacle is that isolation of peptides in food is a difficult task because the peptides are present in complex mixture containing various substances such as acids, free amino acids, sugars and salts (Careri and Mangia, 2003). In the last decade many research efforts have been made to develop techniques and methods for the separation, purification and characterization of food peptides and proteins including Mass Spectrometry (MS) and High Performance Liquid Chromatography (HPLC) (Careri and Mangia, 2003).

1.2 The project

The objective of this project was to study the ACE inactivation ability of different fish protein hydrolysates and concentrate and isolate active peptides from the most active fractions. The results can be used to identify possibilities for utilization of fish proteins and as a base for development of hydrolysates to be used as food ingredients. The project was divided into three phases. In the first phase the aim was to start up columns for fractionation and set up method to measure ACE inhibitory ability of samples. This first phase was thoroughly described in a previous Matís report no. 48-07 (Geirsdóttir and others, 2007). In the second phase a pre-run was made to find the most active fraction of the hydrolysate. In the third and final phase the most promising fraction was made in enough amounts to be able to isolate different fractions on a column. The most active fraction from the column was then characterized with MALDI-ToF Mass Spectrometry.

2. MATERIAL & METHODS

2.1 Raw material

Fish mince from skinless cod fillets was used as a raw material. Samples necessary for the trial minced in one batch, packed in small bags and frozen at -80°C until hydrolyzed. Protein content was 18% according to Kjeldahl measurement (Protein=Nx6.25).

2.2 Hydrolysis

2.2.1 Pre-trial

Sample taken out of the freezer and thawed at room temperature (24°C) until partly thawed and could be taken out of the bag. A solution of protein and cold tap water with 3% protein content measured and homogenized in an Ultra Turrax for 30 seconds at 15.000 rpm. Total volume was around 80 ml. Solution transferred to a reaction holder in a cold room at 10°C. When temperature of the solution had reached 10°C, pH was adjusted to 7.5 with 0.5M NaOH and enzyme added to start the hydrolysis. Cryotin was used as enzyme (NorthIce). Two degree of hydrolysis (DH) were processed, 7.7%DH and 12.5%DH. The enzyme concentration 3% and 5% for each hydrolysis respectively. The pH was kept constant by titration with 0.5M NaOH. Hydrolyzed for 180 minutes to reach the two different DH values and inactivated at 90°C for 10 minutes. Cooled on ice and frozen for fractionation.

2.2.2 Main trial

Same conditions as for pre-trial except 200 ml were hydrolyzed and enzyme concentration was 5%.

3. METHODS

3.1 Hydrolysis

Degree of hydrolysis (DH) was estimated using the Adler-Nissen pH stat method (Adler-Nissen 1986) where DH is the percent of peptide bonds cleaved (h) relative to the total number of bonds per unit weight (h_{tot}) (7.501 mequiv/g). Using this method the amount of base consumed (B) (ml) to maintain a pH is proportional to the hydrolysis equivalents h (mequiv/g of protein) at near neutral or alkaline conditions. Then %DH can be calculated according to:

$$\%DH = \frac{h \times 100}{h_{tot}} = \frac{BN_B}{\alpha h_{tot} \times MP} \times 100$$

where

N_B = normality of the alkaline solution used [M]

. α = average degree of dissociation of α-NH groups (see below)

MP = mass of protein [g]

h_{tot} = total number of peptide bonds = 7,501 mequiv/g

The degree of association α is calculated according to

$$\alpha = 10^{pH-pK} / (1 + 10^{pH-pK})$$

where

pH = pH during hydrolysis and p*K* = dissociation constant.

The p*K* value varies significantly with temperature but is relatively independent of the substrate as such. The p*K* values at different temperatures (T in Kelvin) can be calculated according to

$$pK = 7.8 + \frac{298 - T}{298T} \times 2400$$

3.2 Fractionation

Samples were fractionated using centrifugation, ultra filtration and a column.

3.2.1 Centrifugation

The following steps were performed to fractionate samples

1) Centrifuged at 20.000 x g for 20 minutes at 4°C
2) Filtrated with 0.45µm filter
3) Centrifuged with <30kDa microfilter at 3000xg at 4°C until all liquid was through
4) Centrifuged with <10kDa microfilter at 3000xg at 4°C until all liquid was through
5) Centrifuged with < 5 kDa microfilter at 3000xg at 4°C until all liquid was through

Microfilters from Millipore were used: Centricom Plus-70. Samples collected and frozen for further studies.

3.2.2 Column

To separate different fractions it was decided to use size exclusion techniques. The column (3x103cm column from Bio-Rad) used was packet with Biogel P2 Fine 45-90µm (Bio-Rad Cat No 150-4115) according to manufactures specifications. The columns were connected in series of three to increase the column volume. Sample was concentrated in a rotovapor to thick solution, solubilized in 5% (v/v) formic acid solution and injected to the column. The column was run at 0.2mL min^{-1} in 5% formic acid with Akta Purifier from GE healthcare. The effluent was monitored at 280 nm. Fractions of 4 mL were collected until no peaks were observed. Samples were pooled according to the elution profile, frozen and freeze dried for further analysis.

3.3 Protein measurements

Protein content in samples was determined by measuring nitrogen in samples with Kjeldahl method (ISO 1997) or Dumas method. The Dumas method was performed by using a macro analyzer vario MAX CN equipment (Elementar Analysensysteme GmbH, Germany). A factor of 6.25 was used to convert nitrogen to crude protein content.

3.4 Electrophoresis

Determination of the molecular weights of the samples was performed by SDS-PAGE analysis using a pre-cast Tris-Tricine gel (16% resolving gel, 4% stacking gel) from Invitrogen. The samples were prepared for the electrophoresis according to the

manufacturer's instructions. The protein content of the sample placed in each well of the gel was adjusted to 50 µg. The electrophoresis was run for 45 min at 80 mA following 40 mA for further 45 min. After the electrophoresis, the gels were stained with Simply Blue™ Safe Stain (Invitrogen) according to manufacturer's instructions. Polypeptide SDS-PAGE molecular weight standards in the size range of 26.625 – 1.423 kDa (BioRad, Hercules, CA, USA) were used for MW estimation.

3.5 ACE inhibitation

The ACE inhibitor activity was performed according to the method described by Shalaby and colleagues (2006) with slight modification. FA-PGG was used as substrate at 0.88 mM of solution in Tris-HCl/NaCl buffer pH 7.5. Angiotensin converting enzyme from rabbit lungs was freshly prepared before measurements, 0.2 U/ml of the enzyme solution was prepared and kept on ice. Microplate reader set (Polarstar Optima, BMG labtech) at 37°C was used. The absorbance at 340 nm was recorded each 45 seconds for 33 minutes, and the slope average over a linear interval between 15 and 33 min was taken as a measure of ACE activity. The ACE activity was expressed as the slope of the decrease in absorbance at 340 nm (Δsample), and the ACE inhibition (%) was calculated according to (1)

$$(1-(\Delta_{sample} / \Delta_{control}) \times 100) \quad (1)$$

Where Δ_{sample} is the slope in the presence of inhibitor and Δ_{control} is the slope in the absence of the inhibitor. The concentration of ACE inhibitor peptide that reduces ACE activity by 50% was defined as IC_{50} value.

3.6 MALDI-ToF Mass Spectrometry

The samples were analyzed via a combination of an Agilent 1200RR HPLC and a Bruker microtof-Q MS(n) with an ESI ionization chamber. The HPLC separation was obtained by employing Milli-Q water with a varying percentage of acetonitrile. Both liquids contained 1% formic acid (V/V). The gradient profile was as follows: 5 min isocratic @ 95% water/5% ACN, decreasing linearly to 86.4% water/13.6% ACN @ 19.9 min, decreasing linearly again to 50%water/50% ACN @ 33 min and finally jumping to 10% water/90% @ 33.1 minute and staying there until @ 40 min (column cleaning). The

column was a Phenomenex Gemini-NX C18 150mmx2.00mm with a 3 um particle size that was re-equilibrated for 4 minutes between runs and held at constant temperature of 30°C. The HPLC flow rate was 0.18 mL/min with the ESI nebulizer gas set at 1.2 bar. Dry gas was held at 8 L/min while the dry temperature was constant at 190°C. The microtof-Q instrument was operated at a setting favoring the mass range of 100 to 900 m/z, but spectra from 50 to 2000 m/z were collected at all times. The mass spectrometer was operated in positive mode and the Bruker ES tuning mix was used to calibrate the instrument prior to analysis as well as being introduced at a steady flow rate during the HPLC-MS analysis via a syringe pump.

4. RESULTS & DISCUSSION

4.1 Pre- trial

4.1.1 Hydrolysis

Hydrolysis graph for the runs can be seen in Figure 2.

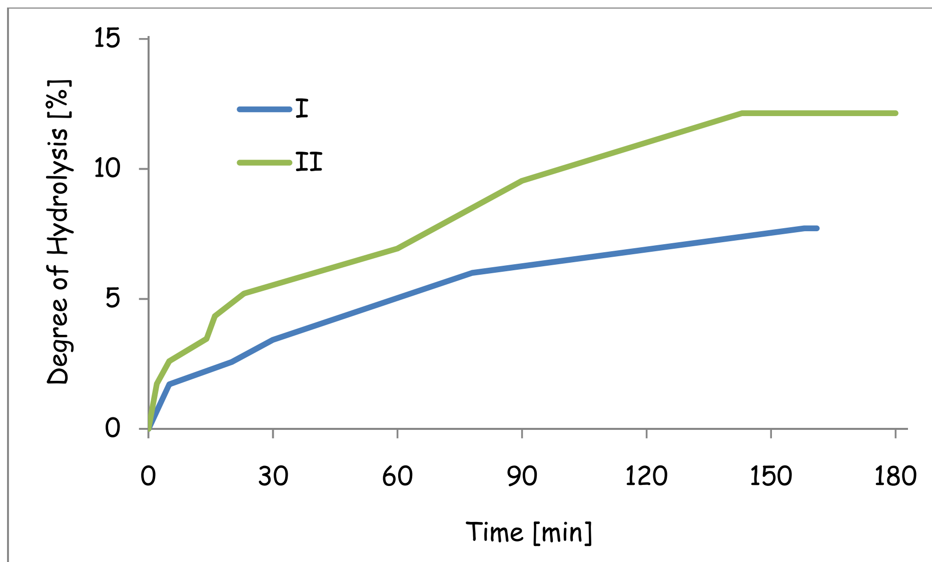

Figure 2. Degree of hydrolysis of cod fillet proteins (3% solution) with Cryotin F at 10°C and pH 7.5. I 3% enzyme, II 5% enzyme.

4.1.2 Fractionation

Time necessary for the centrifugation in the different microfilter were following:

<30 kDa – 45 minutes

<10 kDa – 15 minutes

<5 kDa – 15 minutes.

Samples frozen for further protein and ACE inhibition measurements.

4.1.3 ACE

ACE was measured directly in the fractions from the filtration. The following values were obtained (Table 1).

Table 1. Degree of hydrolysis, protein concentration, ACE inhibitation activity and IC_{50} values for different fractions of two hydrolysates from cod mince using Cryotin F.

Sample	Degree of hydrolysis [%]	Fraction	Protein [mg/mL]	ACE inhibition [%]	IC_{50} [mg/mL]
I-1	7,7	>30 kDa	19,3	75,6	1,7
I-2	7,7	< 30 kDa	17,2	71,8	1,8
I-3	7,7	< 10 kDa	14,0	94,9	1,4
I-4	7,7	< 5 kDa	8,2	84,6	0,1
II-1	12,5	>30 kDa	20,6	94,9	1,4
II-2	12,5	< 30 kDa	19,1	85,9	0,8
II-3	12,5	< 10 kDa	16,3	70,5	0,2
II-4	12,5	< 5 kDa	9,8	78,2	0,1

From the results (Table 1) it was concluded that the fraction containing the smallest peptides (< 5 kDa) and the higher degree of hydrolysis (12.5%DH) had the lowest IC_{50} value and thereby the highest inhibitation activity.

It was therefore decided to reach high degree of hydrolysis and fractions with lowest particles (<5kDa) in the main trial.

4.2 Main trial

Outline for the main trial is shown in Figure 3.

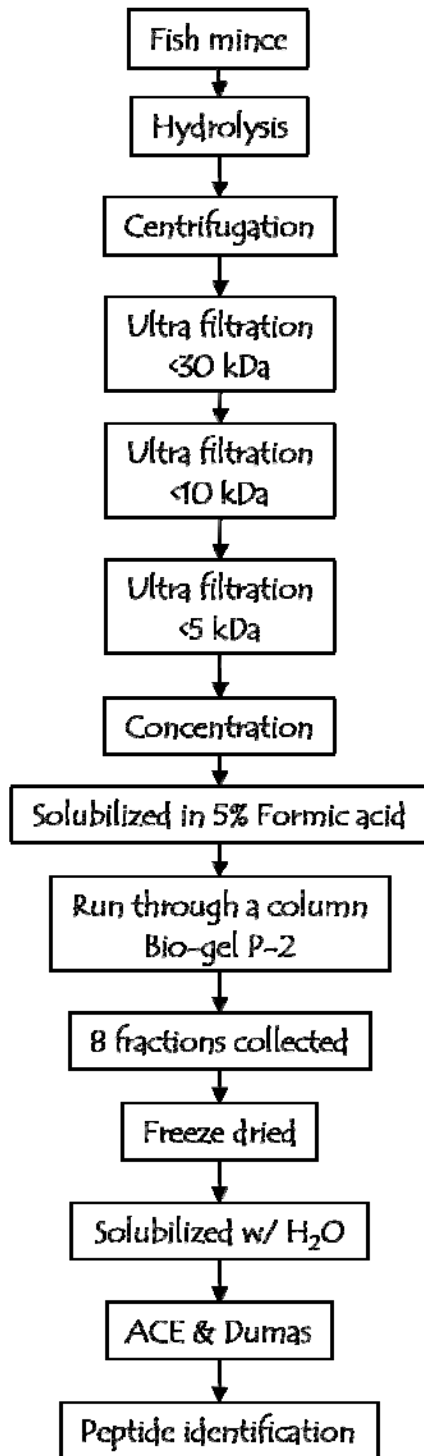

Figure 3. Outline of the main trial.

4.2.1 Hydrolysis

Higher pH was used for hydrolysis in the main trial than in the pre-trial or pH 8 instead of pH 7.5. Higher degree of hydrolysis was obtained or 15% (Figure 4).

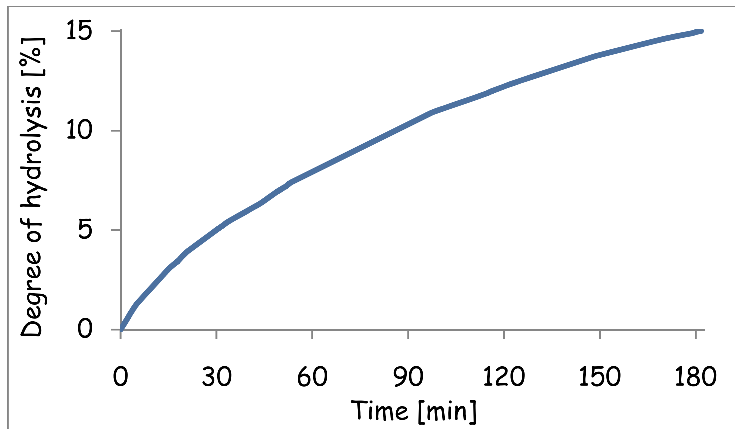

Figure 4. Degree of hydrolysis of cod fillet proteins (3% solution) with 5% Cryotin F at 10°C and pH 8.

4.2.2 Electrophoresis

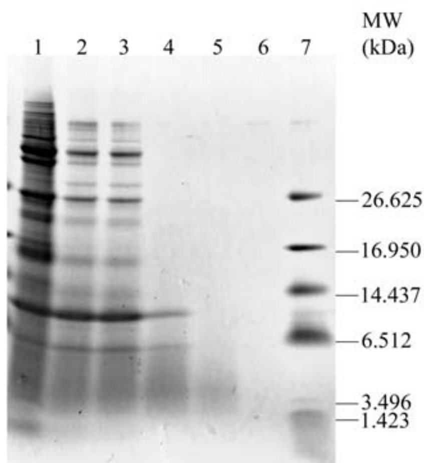

Figure 5. SDS electrophoresis of fractions from 15%DH hydrolysis of cod fillets with Cryotin F. Lane 1 - Unfractionated after hydrolysis, heating, freezing and pH adjustment to 7.5; Lane 2 - Centrifuged at 20.000 x g for 20 minutes at 4°C; Lane 3 - Filtrated with 0.45µm filter; Lane 4 - Centrifuged with <30 kDa microfilter; Lane 5 - Centrifuged with <10 kDa microfilter; Lane 6 - Centrifuged with < 5 kDa microfilter

Electrophoresis (Figure 5) showed that centrifugation and filtration with 0.45µm filter did not change the composition of the proteins, mainly the protein content. Furthermore it shows that the cut off value from the manufacturer of the ultrafiltration cups should only

be taken as an indication for the molecular mass of the samples after filtration whereas the <30 kDa filter removed many proteins below the 30 kDa mark. The 10 kDa filter is doing its job and protein fractions up to 10 kDa can be seen. No bands are visible for the <5 kDa fraction indicating the presence of low MW peptides which could not be detected using SDS-PAGE.

Previous results as well as results from the pre-trial (Table 1) indicate that the smallest peptides show the highest ACE inhibitation activity (Raghavan and Kristinsson, 2009). That fraction was therefore concentrated and fractionated further on a column.

4.2.3 Fractionation

Eight different fractions were collected from the column. The total figure is shown in Figure 6 and parts of the fractions in Figures 6a to 6e. The fractions were freeze dried, solubilized in water and sent to protein measurements, ACE and MALDI-ToF analyzes.

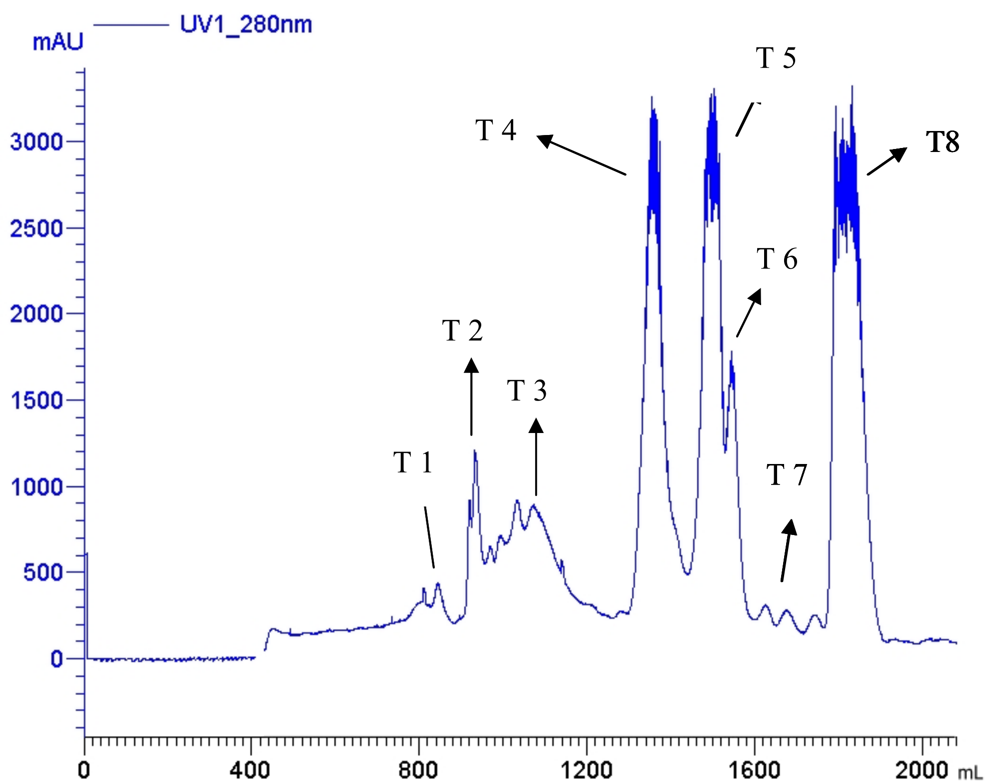

Figure 6. Peaks at 280 nm from a P-2 Colum. Location of the different fractions (T) is marked on the figure. More accurate fractions can be seen on figures 5a-e.

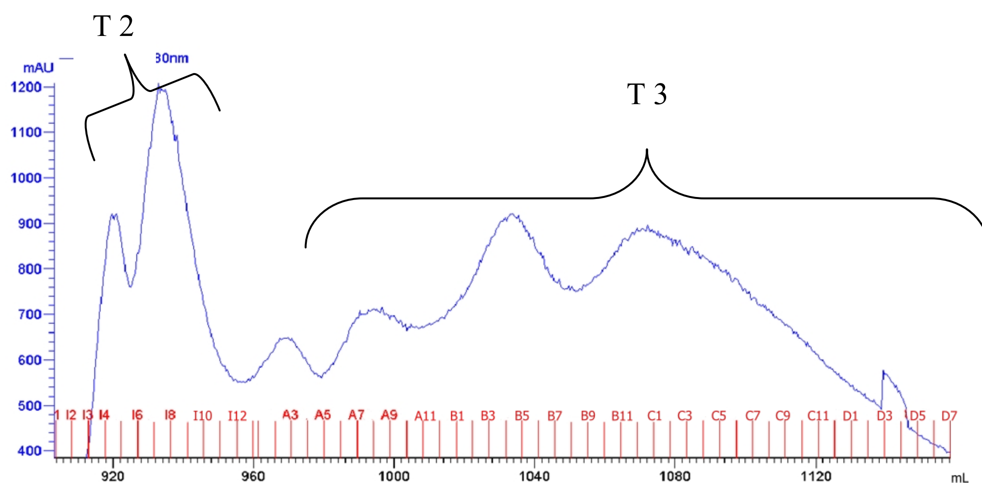

Figure 6a. Part picture of Fractions 2 (T2) and 3 (T3).

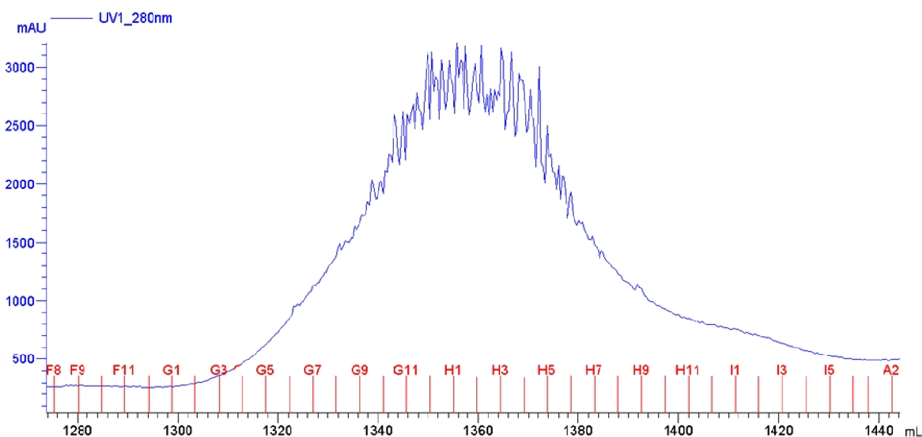

Figure 6b. Part picture of Fraction 4.

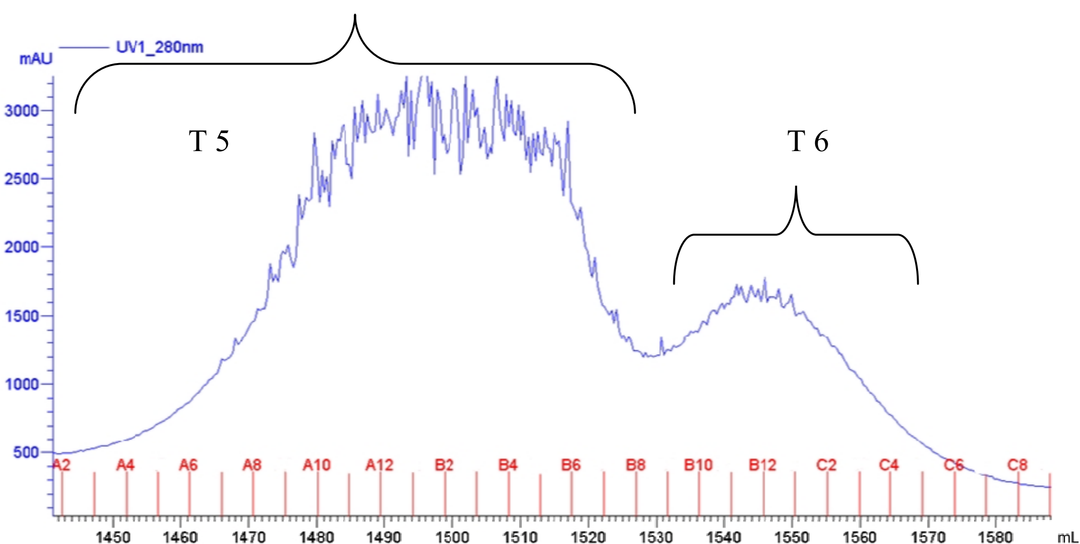

Figure 6c. Part picture of Fractions 5 (T5) and 6 (T6).

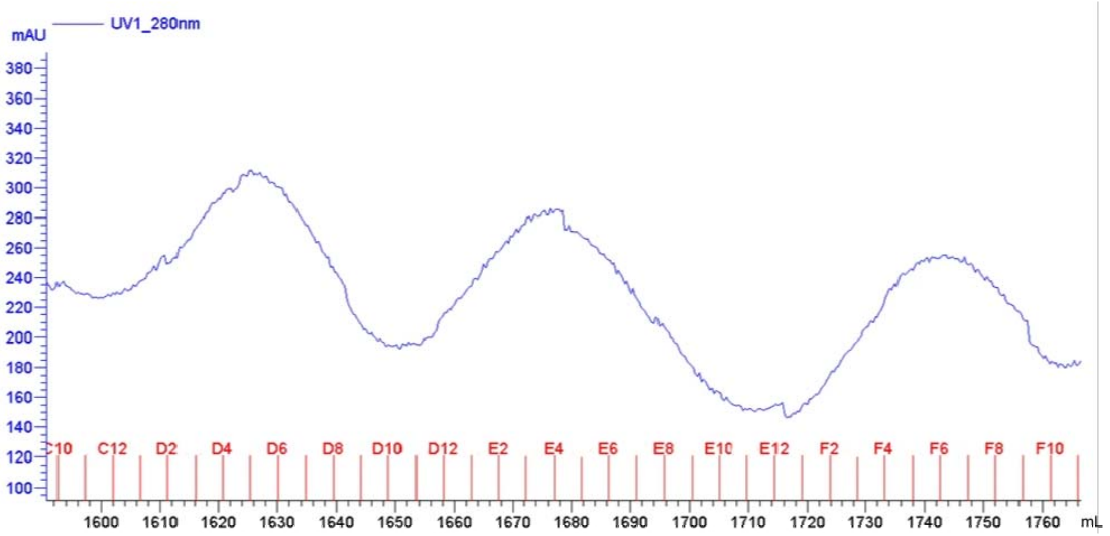

Figure 6d. Part picture of Fraction7.

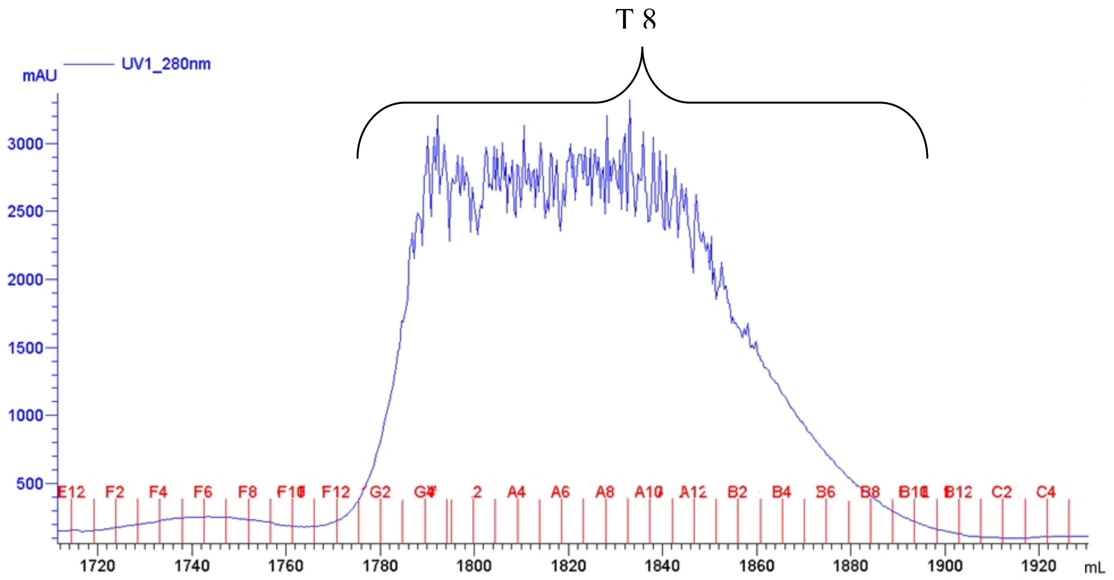

Figure 6e. Part picture of Fraction 8 (T8).

4.2.4 ACE

During hydrolysis a wide variety of large, medium and small peptides are generated, depending on the enzyme specificity and the extent of hydrolysis. All fractions displayed an ability to significantly inhibit ACE except for fraction 6 (Figure 7a).

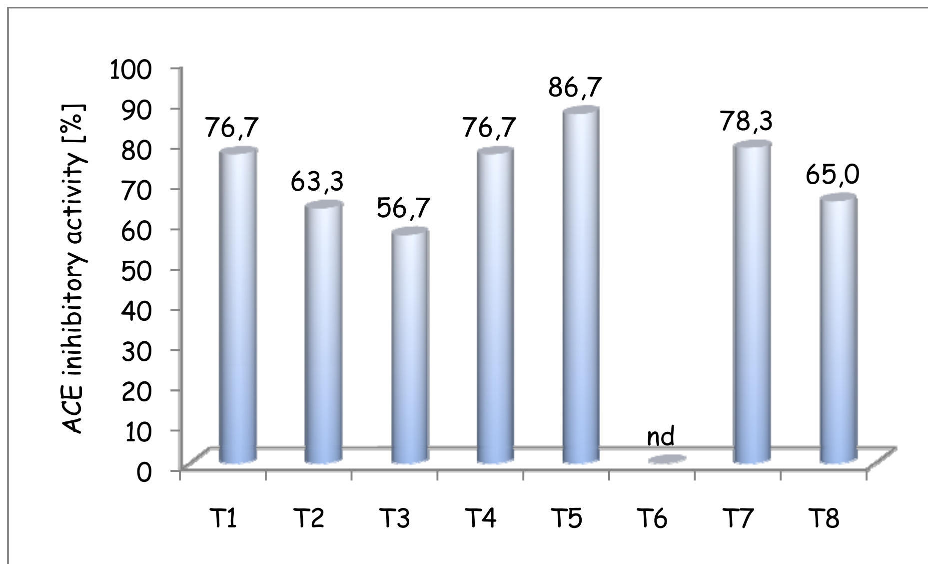

Figure 7a. ACE inhibitation activity of 8 different fractions collected from a column.

IC_{50} values are better for comparison (Figure 7b) were fraction 5 had the highest activity and fraction 1 the lowest. Fraction 5 had over 5 times higher ACE inhibitor activity compared to fraction 1.

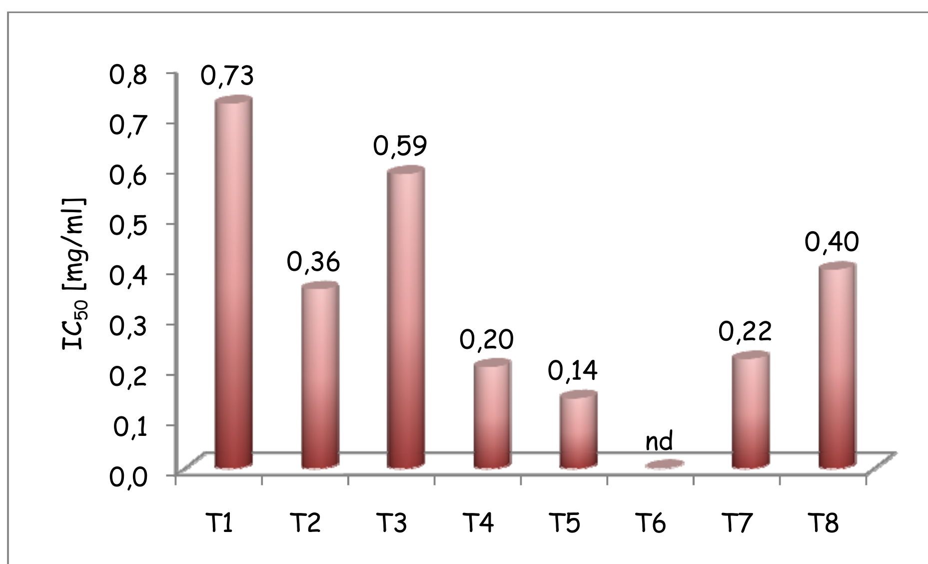

Figure 7b. IC_{50} values of 8 different fractions collected from a column.

Bougatef and others (2008) studied sardine hydrolysates with different enzymes giving 5 – 11%DH with reported IC_{50} values from 1.2-7.4 mg/ml which is in line with what the biggest fractions of the 7%DH showed in the pre-trial (Table 1). By fractionation the IC_{50} values of the sardine hydrolysates were improved to 0.81 mg/ml. In similar manner Je and colleagues (2004) obtained IC_{50} value at 0.5 mg/mL for peptides from Alaska Pollack frame protein hydrolysates. Furthermore, even lower values have been reported for ultrafiltrated cod frame protein hydrolysates, or around 0.01 mg/ml (Jeon and others 1999). It's obvious that the ACE inhibition of our fractions is similar to what other researchers have found. Fraction 5 is of special interest. It has the lowest IC_{50} value indicating high ACE inhibitation activity (Figure 7b).

4.2.5 MALDI-ToF

MALDI-ToF analyses were made of the fractions (Figures 8a-f). It was not possible to measure fraction nr. 3 whereas it did not dry. The enzyme used (Cryotin F) is kept in glycerol, it might be that the glycerol was in fraction 3 resulting in drying problems. No signal was apparent for fraction 4.

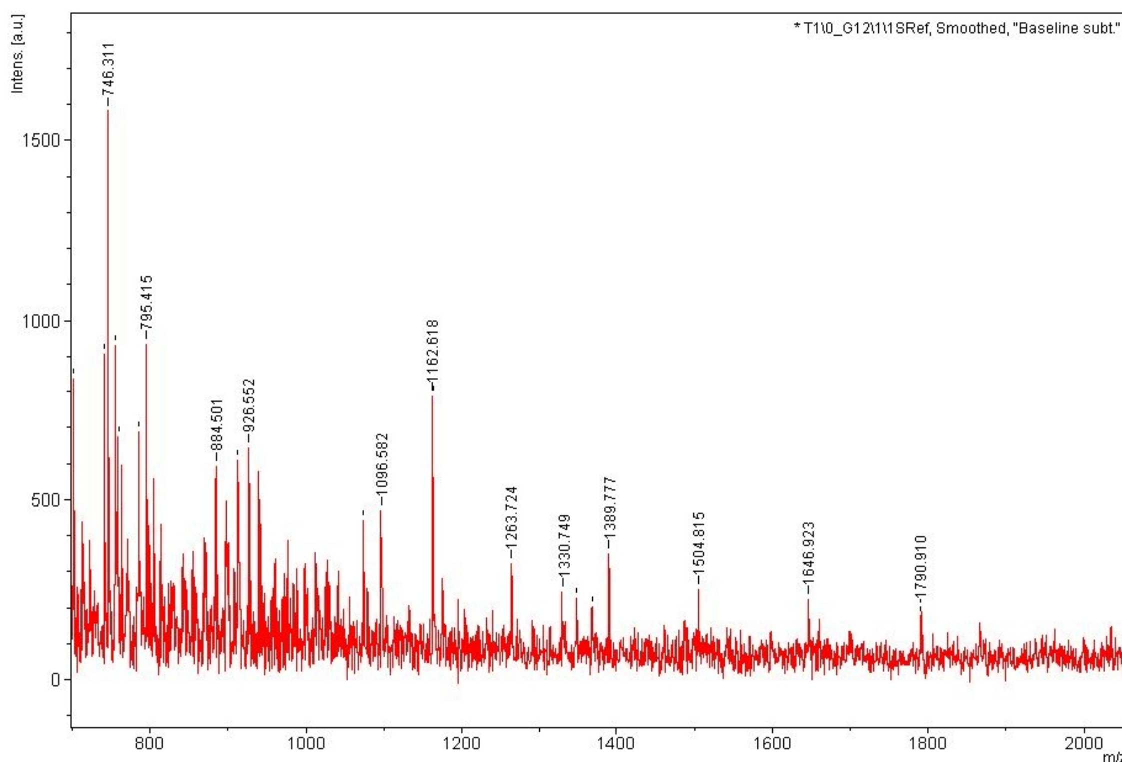

Figure 8a. MALDI-ToF spectra of fraction 1.

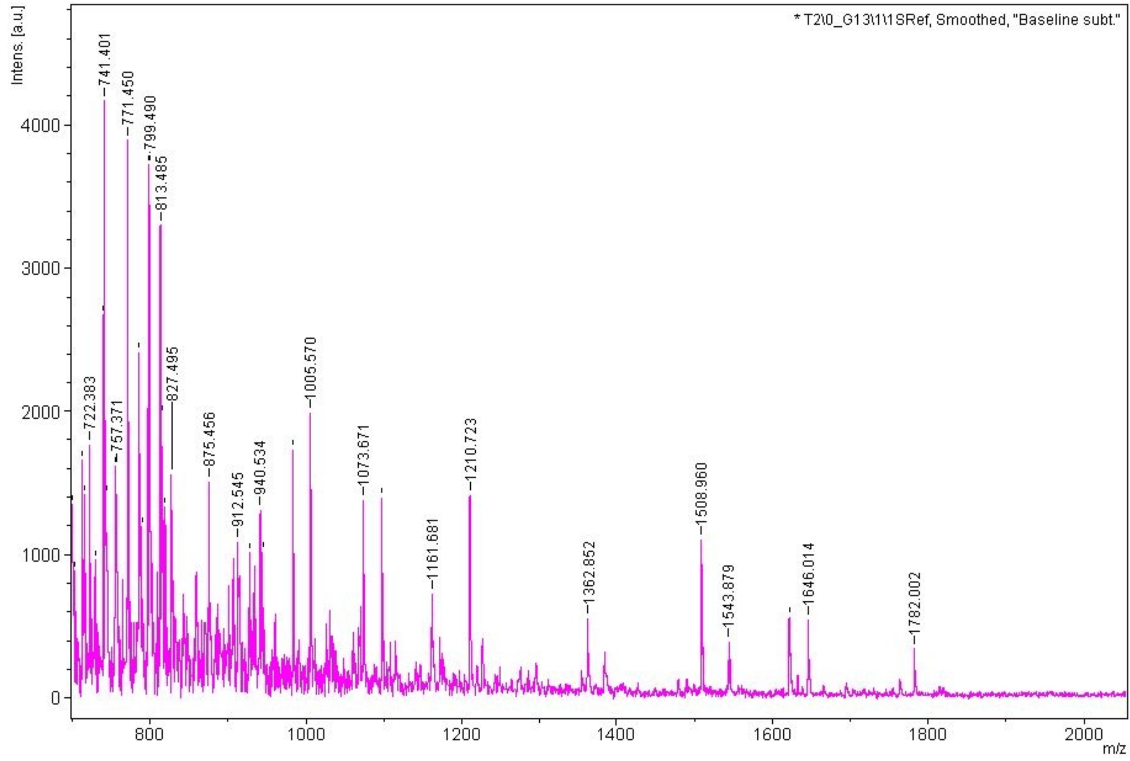

Figure 8b. MALDI-ToF spectra of fraction 2.

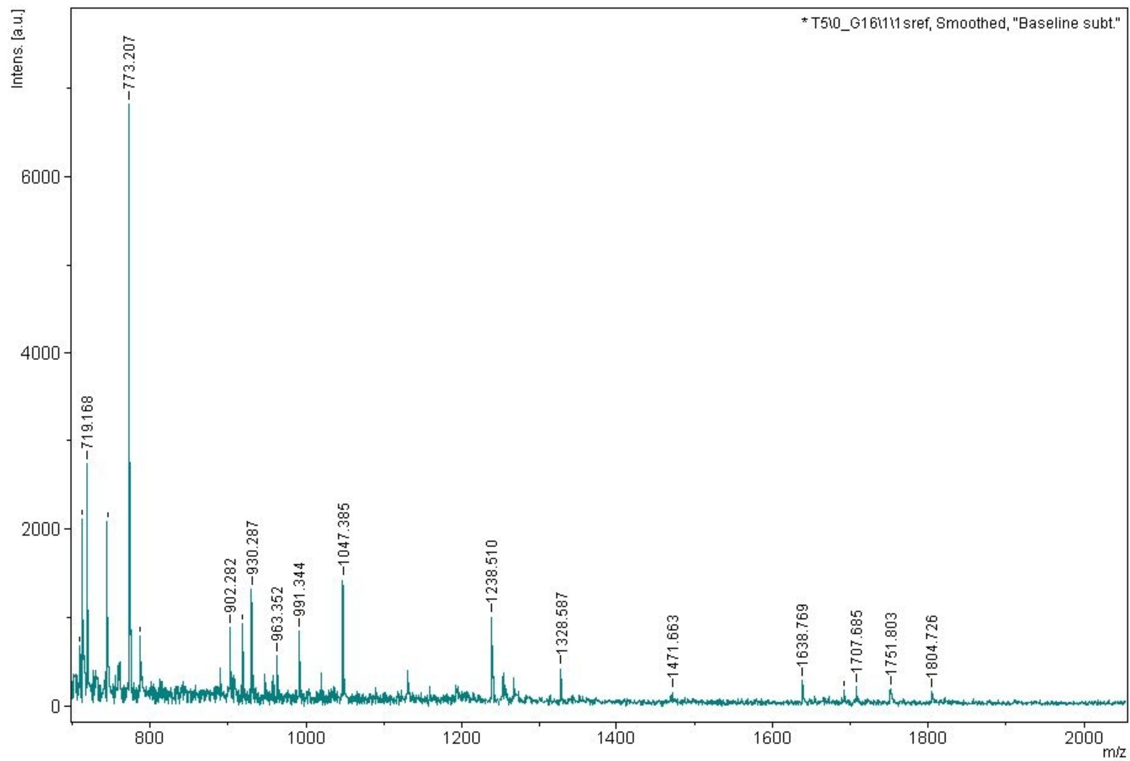

Figure 8c. MALDI-ToF spectra of fraction 5.

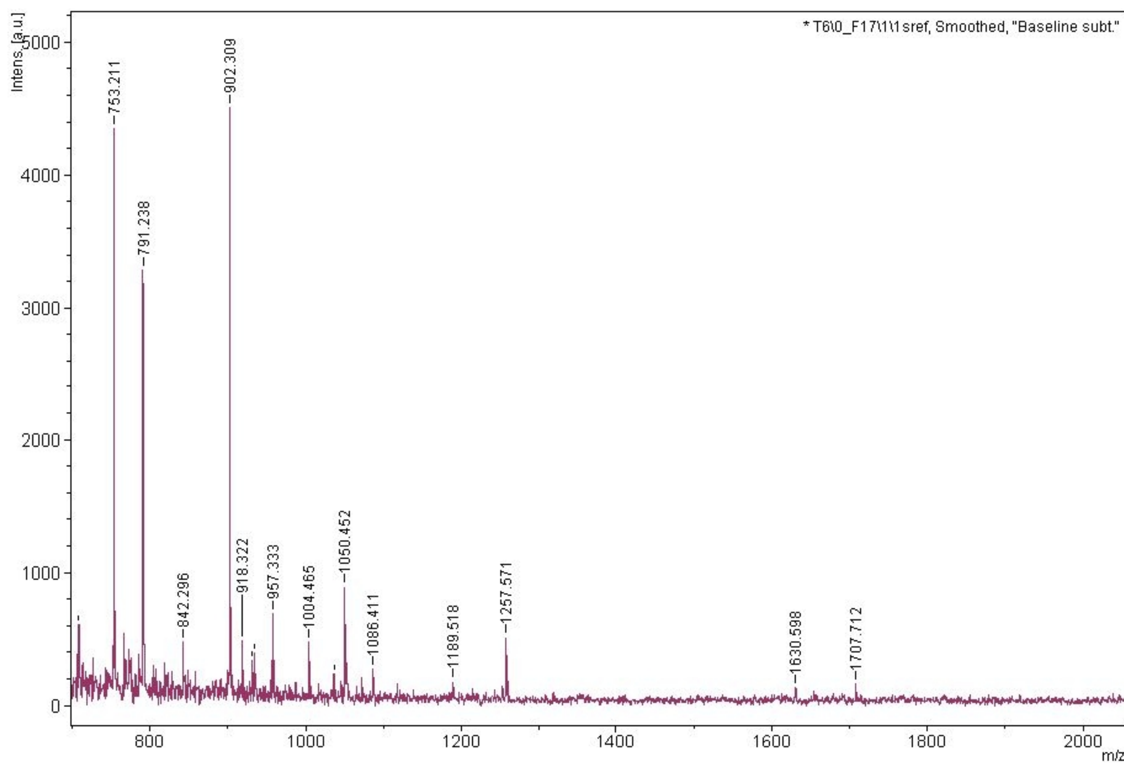

Figure 8d. MALDI-ToF spectra of fraction 6.

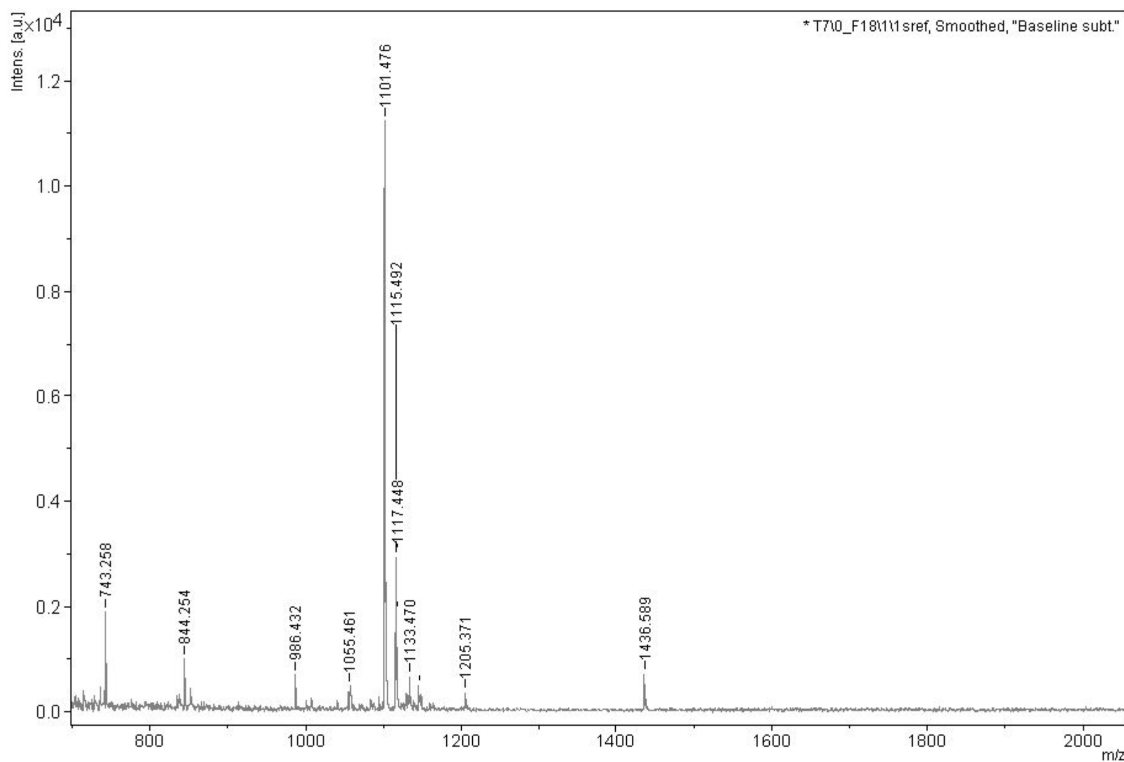

Figure 8e. MALDI-ToF spectra of fraction 7.

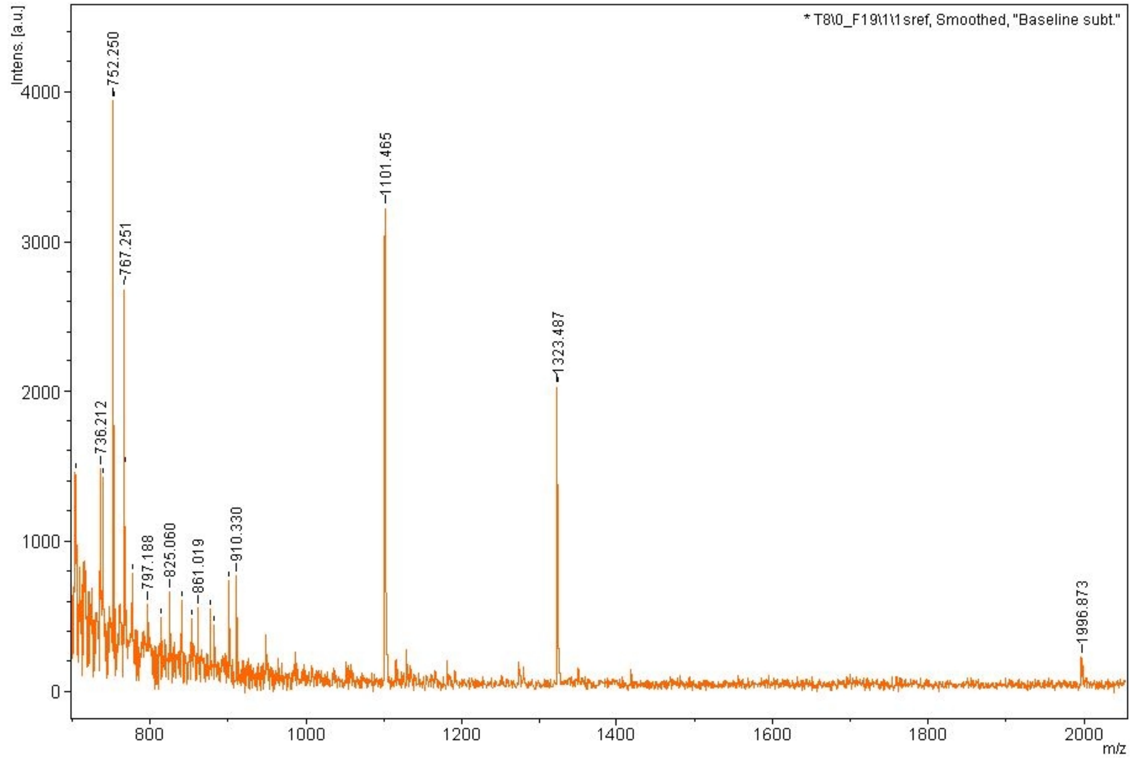

Figure 8f. MALDI-ToF spectra of fraction 8.

Fractions 1 and 2 show to be including a mixture of many peptides. Fraction 7 includes mainly one peptide. Furthermore fractions 5 and 6 include a mixture of small peptides. Whereas fraction 5 showed the highest activity that sample was further analyzed by running them through HPLC and qtof MALDI-TOF (Figure 9 & Table 2).

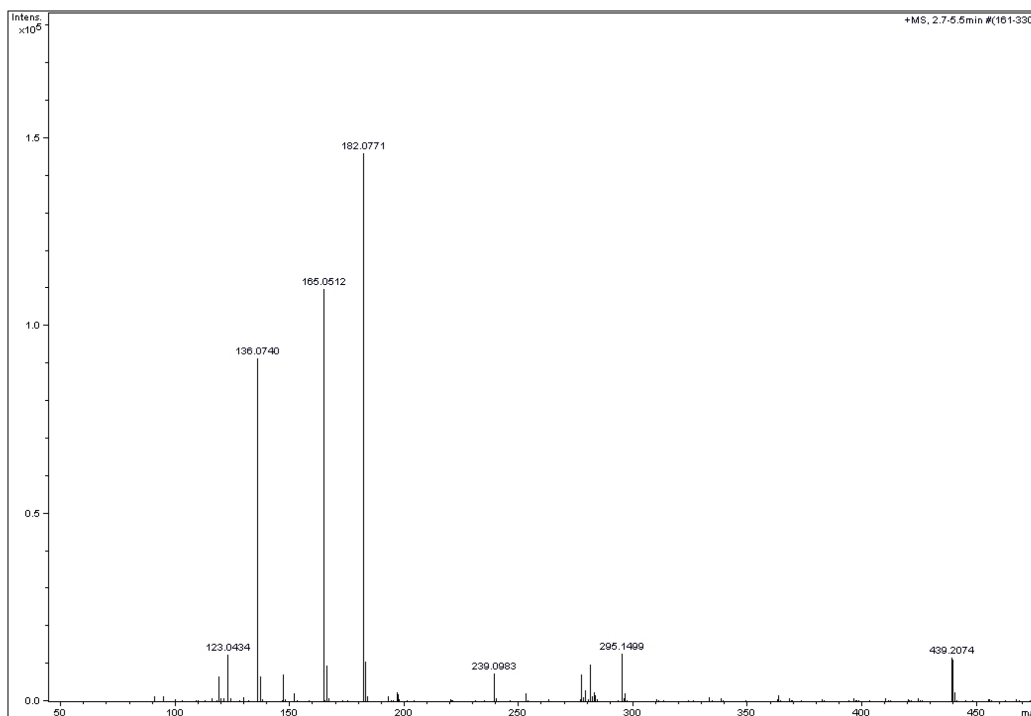

Figure 9. qtof MALDI-ToF spectra of fraction 5. For identification of peaks see table 2.

Table 2. List of peptides analyzed with MALDI-ToF in fraction 5.

Mass [Da]	Area [%]	Peptide	Comment
182.1	56%	Y	consistent with Y
281.1	12%	FD	water loss consistent with D, 166 consistent with F (y1 ion)
239.1	8%	YG	182 consistent with Y
295.1	5%	EF	water loss consistent with E, no 182 peak, total mass also consistent with YI/L, need ms/ms for confirmation
295.2	4%	YI/L	no water loss, 182 consistent with Y, total mass also consistent with EF, need ms/ms for confirmation
253.1	2%	YA	mass also consistent with FS and possibly HP, need ms/ms for confirmation
283.1	2%	YT	only dipeptide consistent with mass
281.1	2%	YV	182 consistent with Y?, total mass also consistent with FD, need ms/ms for confirmation
295.2	1.3%	-	total mass consistent with EF and YI/L
295.2	1.1%	-	water loss but also 182 consistent with Y, no known dipeptide with total mass that fulfills both
439.2	0.2%	-	no known dipeptide mass
510.2	0.5%	-	no known dipeptide mass

At the University of Olsztyn in Poland an online database has been created named BioPep where results from different researches on bioactive properties of peptides have been collected at www.uwm.edu.pl/biochemia/index_en.php. The peptides listed in table 2 were run through the database. Phenylalanine-Aspartic acid (FD) is the most abundant dipeptide in the fraction (Table 2). This peptide has not a listed bioactivity according to the database. On the other hand the next peptide Tyrosine–Glycine (YG) is known for its ACE inhibition activity that might explain the high activity of fraction 5.

Similar analyses were performed for fraction 7 (Table 3).

Table 3. List of peptides analyzed with MALDI-ToF in fraction 7.

Mass [Da]	Area [%]	Peptide	Comment
297.1	16.2%	YD	182 consistent with Y, total mass consistent with YD and also FM
302.2	15.5%	PW	Only dipeptide consistent with mass, but LC peak not indicative of a peptide
293.0	11.4%	YE	M+H-H2O
311.1	8.0%	YE	water loss consistent with E, 182 consistent with Y
318.2	6.3%	I/LW	205 consistent with W, only dipeptide consistent with mass
391.2	5.1%	WW	205 consistent with W, only dipeptide consistent with mass
221.1	3.9%	AM/CV	mass also consistent with DS and TT but no water loss so unlikely
276.1	3.2%	WA	205 consistent with W, total mass also consistent with EQ, EK and RT, very little water loss so E is unlikely, no ammonia loss so K is unlikely
318.2	2.8%	I/LW	205 consistent with W, only dipeptide consistent with mass
304.2	2.7%	WV	205 consistent with W, total mass also consistent with ER
448.2	2.6%	-	no known dipeptide mass
589.2	1.6%	-	no known dipeptide mass
302.2	1.4%	PW	Only dipeptide consistent with mass, 205 (W ion) not observed

In fraction 7 the most apparent dipeptides are Tyrosin-Glutamic acid (YE) and Tyrosine-Aspartic acid (YD) and but also many peptides containing Tryptophan (W). In BioPep ACE inhibition of YD or YE is not mentioned but many dipeptides including W on the other hand show this activity. Osajimi and collegues have on the other hand patent on production of the dipeptide Valine-Tyrosine (VY) from sardines (Osajima and others 2005; 2006 a, b).

As mentioned previously activity measured in a test tube is not the same as effect in the human body. The peptides need to get through the digestion track and be taken up in the blood stream to have a bioactive effect. By using simulated gastrointestinal digestion studies it has been shown that some peptides are stable under simulated gastrointestinal conditions and are able to reach the blood making these peptides more likely to be effective as antihypertensive (Foltz and others, 2007). Its particularly C-terminal Pro- and Pro-Pro-containing peptides that have those properties. Furthermore by giving humans yogurt containing those peptides and by conducting a meta-analysis of randomized controlled trials to assess their effects it has been shown that those peptides are present in plasma of human subject (Xu and others, 2008). The peptides obtained in this research did not contain proline.

To be able to confirm the activity measured *in vitro* in this project animal and human studies are necessary.

5. CONCLUSIONS

The aim of this project was to study the activity of fish proteins and isolate, clarify and define peptides with antihypertensive properties. During the project time methods and equipment to be able to do this has been set up at Matis facilities. This includes how to measure ACE inhibition activity as well as filtration and fractionation units to isolate different fractions of peptides. Furthermore by using HPLC and MALDI-ToF analysis in collaboration with the University of Iceland peptides were identified. Some of those peptides have a known ACE inhibition activity but others have not been reported indicating that new promising peptides can be processed.

With this extensive tool box of knowhow, equipment and facilities, development of valuable fish products and nutraceuticals from blood pressure-lowering peptides is possible. Thereby the value of the Icelandic natural resources in the sea can be increased.

6. ACKNOWLEDGEMENTS

This project was funded by the AVS research fund under the Ministry of Fisheries in Iceland (project no. R-047-07). The financing of this work is gratefully acknowledged.

The help and collaboration of Dr. Patricia Yuca Hamaguchi and Dr. Hólmfríður Sveinsdóttir at the Matis biotechnology center at Sauðárkrókur and Jón Óskar Jónsson and Rósa Jónsdóttir at Matis is highly appreciated. Furthermore the input of Dr. Sigurður Víðir Smárason at the University of Iceland and Lárus Þórhallsson MS student is especially thanked.

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