ACE-inhibitory peptides FQPSF and LKYPI identified in *Bacillus subtilis* A26 Hydrolysate of Thornback Ray muscle

Running title: ACE inhibitory peptides in *Raja clavata* muscle

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Abstract

ACE inhibitory peptides have been searched in Thornback ray (*Raja clavata*) muscle hydrolysed with *Bacillus subtilis* A26 peptidases until an hydrolysis degree of 18.35% . The hydrolysate showed an IC_{50} of 0.83 mg/mL. To identify peptides responsible for this activity, the extract was eluted through size-exclusion chromatography and fractions collected. The highest ACE inhibitory activity was found for fractions F2 and F3 which had IC_{50} of 0.42 and 0.51 mg/mL, respectively. These fractions were analysed by nano-liquid chromatography coupled to tandem mass spectrometry (nLC-MS/MS). A total of 131 and 108 peptide sequences mainly derived from actin, myosin heavy chain and procollagen alpha 1 chain proteins were identified in fractions F2 and F3, respectively. FQPSF and LKYPI showed the best results with an IC_{50} of 12.56 and 27.07 µM, respectively. These results prove the potential of thornback ray muscle hydrolysate as a source of ACE inhibitory peptides.

Keywords: ACE inhibitory activity, Thornback ray, *Bacillus subtilis* A26 hydrolysate, proteomics, mass spectrometry.

Introduction

Hypertension is one of the major risk factors for the development of cardiovascular diseases, stroke, and end-stage renal disease. Different health effects have been attributed to food-derived peptides being the lowering of blood pressure the most extensively studied over the past three decades. The angiotensin I converting enzyme (ACE) catalyzes the formation of angiotensin II, a strong vasopressor, from angiotensin I, and inactivates the bradykinin possessing hypotensive activity.

Naturally occurring ACE inhibitory food peptides constitute part of the daily diet but they could also be used in functional foods. In fact, the use of muscle protein derived from meat, fish, or invertebrates to contribute with ACE inhibitory peptides to the diet has been of great interest during the last years. There are some recent published studies of ACE inhibitory peptides identified in fish muscle protein such as flounder fish and tilapia (Huang et al 2015; Ko et al 2016).

Thornback ray is a widely distributed skate (Rajiformes: Rajidae) in the eastern Atlantic, also including the Mediterranean and Black Seas and Indian Oceans of southern Africa. Thornback ray skin's gelatin hydrolysate using proteases derived from *B. subtillis and B. amyloliquefaciens* have been previously described as a potential source of bioactive peptides (Lassoued et al., 2015a). The muscle of thornback ray has been the focus of study for mercury and trace element analysis due to its high amount of fat and the increasing safety concern in society (Farrugia et al, 2015). However, recent studies emphasise the positive benefits of this type of fish as a source of good quality proteins based on the balance of omega-3 fatty acid benefits (Alfonso et al, 2013). Also the curent interest on natural bioactive peptides derived from different food hydrolysates have influence recent researches of thornback ray (Pihlanto and Korhonen, 2015). Thus, a recent study concluded that B. subtillis A26 proteases hydrolysates of thornback ray

resulted to be the most active ACE-inhibitory between a crude alkaline protease extract, alcalase, and neutrase enzymes (Lassoued et al., 2015b). Our goal in the present study is the hydrolysis of thornback ray muscle by *Bacillus subtilis* A26 proteases and identification by tandem mass spectrometry (nLC-MS/MS) those released peptides exerting ACE inhibitory activity. Thus, this study describes for the first time the sequence of new peptides from five amino acids size with their respective ACE inhibitory activity.

Experimental

Materials

Fresh thornback rays were obtained in fish market (Sfax, Tunisia). Samples were immediately packaged and kept in ice to the laboratory in less than 30 min. Muscles were used immediately or maintained at −80 °C during less than three months.

Proteolytic enzymes

Crude enzyme preparation from *Bacillus subtilis* A26 was prepared as described by Lassoued et al, (2015a). Alkaline protease activity was assayed against casein as substrate following the method described by Brikia, Hamdib, and Landoulsi, (2016).

Preparation of thornback ray muscle hydrolysate (TRMH)

In order to inactivate endogenous enzymes, Thornback ray muscle (500g) was first cooked in 1L distilled water at 90°C for 20 min. The cooked muscle sample was then homogenized in a Moulinex® blender for about 3min. Then, the hydrolysis started by adding the crude enzyme preparation from *B. subtilis* A26 at a 3:1 (U/mg) enzyme/protein ratio. During the reaction, pH was kept at 8.0 and temperature set at 40°C. The hydrolysis was stopped by heating at 90°C for 10 min and then cooled down to room temperature. The solution was centrifuged at 5,000×g for 20 min and the supernatant was freeze-dried (Bioblock Scientific, France) and stored at -20 °C until use. The pH-stat method was used to determine the degree of hydrolysis. Analysis was done by triplicate.

Determination of ACE inhibitory activity

The assay of ACE inhibitory activity was performed as described by Escudero, Mora and Toldrá (2014) using the fluorescent substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-Phe-(NO2)-Pro). ACE inhibition is expressed as percentage and the assays were done by triplicate.

Size-exclusion chromatography

The freeze-dried hydrolysate (1 g), was dissolved in 5 mL of bidistilled water, and eluted with distilled water at a flow rate of 27 mL/h through a Sephadex G-25 gel filtration column (2.9 cm × 53 cm). The elution profile was measured at 214, 254 and 280 nm and 4 mL fractions were collected automatically selecting those showing ACE inhibitory activity that were pooled together and freeze-dried (Bioblock Scientific, France).

Peptide identification by tandem mass spectrometry

Peptide identification was done using a nano-liquid chromatography system (Eksigent of AB Sciex, CA) coupled to a quadrupole-time-of-flight (Q-ToF) system (TripleTOF® 5600+, AB Sciex Instruments, Framingham, MA) equipped with a nano-electrospray ionization source (nano-ESI). Systems parameters were adjusted as previously published in Lassoued et al. (2015a).

Regarding the spectra analysis, the peak list generation and database search for the identification of the peptides were done using Mascot Distiller v2.4.2.0 software (Matrix Science, Inc., Boston, MA). Database search was done in 'Other Chordata' taxonomy using NCBInr database with no modifications and a tolerance of 100 ppm in MS and 0.5 Da in MS/MS. All identified sequences were $p<0.05$. BIOPEP database was used in the search of similar sequences previously identified showing ACE inhibitory activity (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).

Peptide Synthesis

Selected ACE inhibitory peptides were synthesized by GenScript Corporation (Piscataway, NJ) and used for assaying their respective *in vitro* ACE inhibition and IC_{50}.

Results and Discussion

Preparation of TRMH

The enzymatic digestion of proteins is a method of choice to generate peptides showing improved nutritional properties and biological activities. The produced biopeptides depend on the protein substrate; the specificity of the enzyme, as well as the conditions of the proteolysis, in particular the degree of hydrolysis.

In this study, thornback ray muscle hydrolysate was prepared by treatment using *B. subtilis* A26 proteases. As revealed by the zymogram, this strain produces at least seven proteases which may enhance protein hydrolysis and produce a hydrolysate with high amounts of low molecular weight peptides (results not shown).

The hydrolysis curve of TRMH after 400 min incubation is reported in Figure 1A. The curve showed the highest rate of hydrolysis during the initial 15 min. The degree of hydrolysis (DH) of TRGH treated with *B. subtilis* A26 proteases (TRGH-A26) was about 18% (Figure 1A).

The ACE inhibitory activity at different concentrations of TRMH-A26 was investigated and reported in Figure 1B. As expected, the activity was concentration dependent and increased with TRMH-A26 concentration. The IC_{50} of muscle hydrolysate was 0.83 mg/mL, whereas that of undigested muscle was 9.66 mg/mL (data not shown). The activity increased considerably, suggesting that ACE inhibitory peptides are encrypted within the sequence of the parent protein. These results are very similar to those previously obtained in thornback ray skin's hydrolysates using *B. subtilis* A26 proteases, which showed an IC_{50} value for ACE inhibitory activity of 0.94 mg/mL (Lassoued et al, 2015a).

Fractionation of TRMH-A26 by gel filtration

Thornback ray muscle hydrolysate was fractionated by using size-exclusion chromatography (Figure 2A) and 4 mL fractions were collected and assayed for ACE-inhibitory activity. Those with higher activity were pooled together to obtain major fractions F1 to F5 (Figure 2A). ACE-inhibitory activity and IC_{50} were assayed in each major fraction (Figure 2B). Fractions F2 and F3 showed the highest ACE inhibitory activity with IC_{50} of 0.423 and 0.509 mg/mL, respectively. Therefore, these fractions were analysed by mass spectrometry for the identification of peptide sequences.

Identification of ACE inhibitory peptides by tandem mass spectrometry

Peptides of selected fractions (F2 and F3) were identified by nano-liquid chromatography and mass spectrometry in tandem (nLC-MS/MS). A total of 131 and 108 peptides derived from different fish muscle proteins were identified from F2 and F3, respectively (Table 1 and 2). Figure 3 shows the distribution in percentages of the identified peptides according to their origin protein.

Main identified peptides are derived from actin (60 and 47% in F2 and F3, respectively). Actin is an important protein in skeletal muscle responsible for cell movement and muscle contraction that helps to maintain the cytoskeleton in skeletal muscle (Mora et al., 2014).

The second protein showing the highest number of identified peptides was myosin heavy chain protein, showing 33 and 20% of the identified peptides in F2 and F3, respectively. Myosin is the primary structural component of thick filaments, consisting of two myosin heavy chains and four light chain subunits. Connective tissue predominantly consists of fibrillar collagen. Thus, type I procollagen alpha 1 chain protein represents the 24% of the identified peptides in F3 fraction. In fact, collagenous residues have

been reported to give rise to biologically active peptides with high ACE-inhibitory capacity (Ketnawa et al., 2016, and Dave et al , 2016).

From the identified peptides, pentapeptides were the most abundant in both fractions (F2 and F3), whereas longer chains (from 11 to 16 amino acids in length) were less abundant. However, di- and tripeptides could not be detected because the mass spectrometry conditions and data analysis tools did not allow their identification.

Many ACE inhibitors are peptides showing a number of amino acids ranging from 2 to 12. Many of the identified peptides could be responsible for the reported activity, and may exhibit different degrees of ACE-inhibitory activity. In fact, biological activities of peptides are related to their amino acid composition, sequence and size (Mora, Aristoy and Toldrá, 2016). The peptides identified in F2 and F3 showed some sequence homology with previously described ACE inhibitory peptides. In fact, some sequence fragments of the peptides identified from myosin heavy chain protein in fraction F2 have been previously described as ACE inhibitors. Thus, RAD sequence from the myosin heavy chain RADIAES peptide was previously described as ACE inhibitory by Babij et al (2014) in whey proteins hydrolyzed with serine protease isolated from Asian pumpkin. Also the myosin heavy chain peptide GVDNPGHPFI shares sequence with the previously described ACE-inhibitory peptide GVDNPGHPF (IC_{50}> 1000 μM), which was identified from Spanish dry-cured ham (Escudero et al., 2013). Similarly, the C-terminal tripeptide EGY identified in actin peptides IYEGY and NVPIYEGY of F3 agrees with the recently described ACE inhibitory LVVDGEGY peptide (Esteve et al., 2015). The tripeptide ASL with an IC_{50} of 102.15 μM (Wu et al., 2015) has also been identified in F3 in ASLEL peptide from myosin heavy chain and in F2 as part of the actin peptide ILASL. This is very interesting as the last three C-terminal amino acids have been described to be highly

responsible for the inhibition of the ACE enzyme. Thus, not only the composition but also the amino acid sequence position is important in the bioactivity of peptides.

The identified peptides were studied based on the requirements for ACE inhibition, and some of them were synthesised and their IC_{50} calculated (see Table 3). Peptides FQPSF, LKYPI and TLKYP containing Pro and an aromatic amino acid on the C-terminal position, showed the highest ACE inhibitory activity with IC_{50} values of 12.56, 27.07 and 170 µM. Also peptide IITNW identified in F3 showed good ACE inhibitory activity with an IC_{50} value of 30.96 µM, followed by IYEGY peptide with an IC_{50} value of 79.42 µM, probably due to the presence of the branched-chain aliphatic amino acids Ile at the N-terminal position and aromatic amino acids in the C-terminal position Trp and Tyr, respectively. The tripeptide FQP has an IC_{50} value of 12µM similar to the peptide FQPSF with IC_{50} of 12.56 µM. Since ACE cleaves dipeptides from the C-terminus of various oligopeptides, it would appear likely that antihypertensive activity of FQPSF might be mediated by FQP or SF. Peptide ESAGIH showed an IC_{50} value of 371.60 µM. The relatively low activity of this peptide is probably due to its high content in hydrophilic amino acids. The reason is that the hydrophilic-hydrophobic balance is relevant for a peptide having access to the active site of ACE. In this sense, a total of nine ACE inhibitory peptides from cuttlefish (*Sepia officinalis*) muscle hydrolysates were identified and antihypertensive effect of them most potent peptide was tested in spontaneously hypertensive rats (Balti et al. 2015).

It is known that intact di- and tripeptides can be actively transported through the small intestine. However, data describing the enteral absorption of intact oligopeptides larger than two or three amino acids are rather conflicting as most of the peptides can be susceptible to proteolytic degradation during gastrointestinal digestion by cell enzymes (Cian et al, 2015; Gallego et al., 2016). However, peptides showing a proline residue at

the C-terminus have been described to be more resistant to enzymatic hydrolysis so this fact suggests that small biactive peptides identified in the muscle hydrolysate of thornback ray showing Pro residue at different positions of the C-terminal site such as FQPSF, TLKYP or LKYPI might be active not only *in vitro* but also *in vivo*, although further analysis would be necessary for confirmation.

Conclusion

Thornback ray muscle was hydrolysed by using *B. subtilis* A26 proteases and most active SEC fractions were analysed by nLC-MS/MS to identify the peptide sequences. Peptides IWHHT, IVGRPR, IVGRPRHQG and GRPRHQG were identified in thornback ray muscle for the first time and had been previously described as ACE inhibitory peptides from dried bonito. Also some of the identified peptides exhibited a partial sequence homology with other ones described as ACE inhibitors. Selected peptides were synthesized and their sequences assayed *in vitro.* Those showing the highest ACE inhibitory activity were FQPSF and LKYPI peptides with IC_{50} values of 12.56 and 27.07μM, respectively. So, thornback ray muscle hydrolysate may constitute a source of ACE peptides.

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Conflict of Interests:

The authors declare that they do not have any potential sources of conflict of interest.

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Table 1

Sequences using mass spectrometry identified in fraction F2 obtained from size-exclusion chromatography.

Protein Name	Observed[a] (*m/z*)	Expected[b] (Mr)	Charge[c] $[M-H]^+$	Calculated[d] (Mr)	P_0	Sequence	P_f	Acc.Number[f] NCBInr
Actin	420.29	419.29	1	419.20	M	ATAAS	S	gi|392873934
	436.25	435.24	1	435.20	A	TAASS	S	gi|392873934
	223.64	445.26	2	445.25	W	IGGSI	L	gi|392873934
	488.27	487.26	1	487.26	R	LDLAG	R	gi|392873934
	490.29	489.28	1	489.28	G	SILAS	L	gi|392873934
	495.24	494.24	1	494.14	V	CDNGS	G	gi|392873934
	502.32	501.32	1	501.32	Q	AVLSL	Y	gi|392873934
	516.34	515.33	1	515.33	R	GILTL	K	gi|392873934
	516.34	515.33	1	515.33	S	ILASL	S	gi|392873934
	520.28	519.27	1	519.27	R	AVFPS	I	gi|392873934
	536.26	535.25	1	535.25	G	VMVGM	G	gi|392873934
	556.28	555.27	1	555.27	A	GIHET	T	gi|392873934
	559.36	558.35	1	558.35	K	RGILT	L	gi|392873934
	562.24	561.24	1	561.24	C	DNGSGL	V	gi|392873934
	603.37	602.36	1	602.36	R	TTGIVL	D	gi|392873934
	307.15	612.29	2	612.29	M	ESAGIH	E	gi|392873934
	619.31	618.30	1	618.30	A	LAPSTM	K	gi|392873934
	311.18	620.35	2	620.35	L	TLKYP	I	gi|392873934
	626.29	625.28	1	625.26	R	DLTDY	L	gi|392873934
	630.32	629.31	1	629.37	A	IQAVLS	L	gi|392873934
	631.34	630.33	1	630.33	C	DIDIR	K	gi|392873934
	317.20	632.39	2	632.39	T	LKYPI	E	gi|392873934
	640.30	639.29	1	639.29	Q	IMFET	F	gi|392873934
	347.18	692.34	2	692.34	K	IWHHT	F	gi|392873934
	233.15	696.42	3	696.44	S	IVGRPR	H	gi|392873934
	755.36	754.36	1	754.36	D	LTDYLM	K	gi|392873934
	381.72	761.43	2	761.43	T	LKYPIE	H	gi|392873934
	385.69	769.37	2	769.37	L	TDYLMK	I	gi|392873934
	386.68	771.35	2	771.35	F	AGDDAPRA	V	gi|392873934
	791.39	790.38	1	790.39	H	NVPIYEG	Y	gi|392873934
	404.22	806.42	2	806.43	V	GRPRHQG	V	gi|392873934
	412.17	822.32	2	822.32	N	WDDMEK	I	gi|392873934
	417.21	832.40	2	832.39	Y	VGDEAQSK	R	gi|392873934
	420.20	838.38	2	838.38	A	GRDLTDY	L	gi|392873934
	420.22	838.42	2	838.42	E	TLFQPSF	I	gi|392873934
	421.20	840.38	2	840.38	T	FNVPAMY	V	gi|392873934
	432.25	862.48	2	862.48	L	TLKYPIE	H	gi|392873934
	440.21	878.41	2	878.41	R	VAPEEHPT	L	gi|392873934
	297.83	890.47	3	890.47	T	IGNERFR	C	gi|392873934
	451.25	900.48	2	900.50	Q	VITIGNER	F	gi|392873934
	453.21	904.40	2	904.40	A	GFAGDDAPR	A	gi|392873934
	460.22	918.42	2	918.42	G	FAGDDAPRA	V	gi|392873934
	461.73	921.44	2	921.44	T	MYPGIADR	M	gi|392873934
	469.19	936.36	2	936.36	T	NWDDMEK	I	gi|392873934

	477.73	953.45	2	953.45	H	NVPIYEGY	A	gi|392873934
	488.73	975.44	2	975.44	K	AGFAGDDAPR	A	gi|392873934
	488.73	975.44	2	975.44	A	GFAGDDAPRA	V	gi|392873934
	491.78	981.55	2	981.55	L	LTEAPLNPK	A	gi|392873934
	496.75	991.49	2	991.50	R	VAPEEHPTL	L	gi|392873934
	499.75	997.48	2	997.48	R	DLTDYLMK	I	gi|392873934
	510.30	1018.58	2	1018.58	S	IVGRPRHQG	V	gi|392873934
	512.26	1022.50	2	1022.50	D	LAGRDLTDY	L	gi|392873934
	525.73	1049.45	2	1049.45	T	NWDDMEKI	W	gi|392873934
	527.25	1052.48	2	1052.48	T	MYPGIADRM	Q	gi|392873934
	546.26	1090.52	2	1090.51	T	HNVPIYEGY	A	gi|392873934
	548.32	1094.63	2	1094.63	T	LLTEAPLNPK	A	gi|392873934
	567.79	1133.56	2	1133.56	S	YELPDGQVIT	I	gi|392873934
	576.26	1150.50	2	1150.50	I	ITNWDDMEK	I	gi|392873934
	585.29	1168.57	2	1168.57	T	GIVLDSGDGVTH	N	gi|392873934
	391.89	1172.65	3	1172.66	K	IIAPPERKYS	V	gi|392873934
	605.77	1209.52	2	1209.52	W	DDMEKIWHH	T	gi|392873934
	610.84	1219.66	2	1219.66	D	IDIRKDLYAN	N	gi|392873934
	611.30	1220.59	2	1220.59	K	SYELPDGQVIT	I	gi|392873934
	632.80	1263.58	2	1263.58	G	IITNWDDMEK	I	gi|392873934
	428.22	1281.65	3	1281.64	L	AGRDLTDYLMK	I	gi|392873934
	646.32	1290.63	2	1290.62	G	VTHNVPIYEGY	A	gi|392873934
	656.29	1310.57	2	1310.57	W	DDMEKIWHHT	F	gi|392873934
	675.33	1348.64	2	1348.64	G	FAGDDAPRAVFPS	I	gi|392873934
	689.34	1376.67	2	1376.66	G	IITNWDDMEKI	W	gi|392873934
	466.21	1395.61	3	1395.60	N	WDDMEKIWHH	T	gi|392873934
	749.34	1496.66	2	1496.65	N	WDDMEKIWHHT	F	gi|392873934
	504.22	1509.65	3	1509.65	T	NWDDMEKIWHH	T	gi|392873934
	782.38	1562.75	2	1562.74	G	IITNWDDMEKIW	H	gi|392873934
	537.91	1610.70	3	1610.69	T	NWDDMEKIWHHT	F	gi|392873934
	567.61	1699.81	3	1699.80	G	IITNWDDMEKIWH	H	gi|392873934
	861.89	1721.76	2	1721.75	L	DSGDGVTHNVPIYEGY	A	gi|392873934
	460.22	1836.86	4	1836.86	G	IITNWDDMEKIWHH	T	gi|392873934
	485.49	1937.91	4	1937.91	G	IITNWDDMEKIWHHT	F	gi|392873934
Myosin Heavy	238.16	474.30	2	474.21	Y	AGNVD	Y	gi|38347761
Chain	238.16	474.30	2	474.28	G	AGKTV	N	gi|38347761
	488.27	487.26	1	487.26	V	LDIAG	F	gi|38347761
	502.32	501.31	1	501.28	Q	ELLGA	T	gi|38347761
	508.27	507.27	1	507.27	I	VVAGY	R	gi|38347761
	517.28	516.28	1	516.30	K	GGKKQ	L	gi|38347761
	263.14	524.27	2	524.26	K	KGSSF	Q	gi|38347761
	268.66	535.30	2	535.26	D	IAGFE	I	gi|38347761
	305.17	608.33	2	608.33	K	SRVTF	Q	gi|38347761
	623.33	622.33	1	622.26	R	EAEFQ	K	gi|38347761
	211.12	630.33	3	630.33	S	KANAEV	A	gi|38347761
	324.17	646.32	2	646.32	K	ELEEK	M	gi|38347761
	669.36	668.35	1	668.35	G	ALFATF	A	gi|38347761
	338.16	674.30	2	674.30	K	LYDQH	L	gi|38347761
	352.19	702.37	2	702.37	E	TDAIQR	T	gi|38347761

	705.34	704.33	1	704.32	S	AQIEMN	K	gi|38347761
	238.16	711.46	3	711.34	D	KACFLM	G	gi|38347761
	376.18	750.35	2	750.35	R	EAEFQK	L	gi|38347761
	381.18	760.35	2	760.37	E	RADIAES	Q	gi|38347761
	788.38	787.37	1	787.37	L	GEQIDNL	Q	gi|38347761
	396.21	790.40	2	790.40	R	QEAPPHI	F	gi|38347761
	396.68	791.35	2	791.35	T	SAQIEMN	K	gi|38347761
	422.23	842.45	2	842.45	R	VQLELNQ	V	gi|38347761
	433.23	864.44	2	864.39	I	MSILEEQ	C	gi|38347761
	435.21	868.40	2	868.40	Y	DYPFISQ	G	gi|38347761
	458.20	914.38	2	914.38	H	YAGNVDYN	I	gi|38347761
	469.68	937.35	2	937.35	K	MEGDLNEM	E	gi|38347761
	469.74	937.47	2	937.47	R	QEAPPHIF	S	gi|38347761
	487.25	972.49	2	972.49	H	RLDEAEQI	A	gi|38347761
	498.24	994.47	2	994.47	K	YETDAIQR	T	gi|38347761
	508.72	1015.43	2	1015.43	T	TNPYDYPF	I	gi|38347761
	513.26	1024.50	2	1024.50	R	QEAPPHIFS	I	gi|38347761
	526.72	1051.43	2	1051.44	G	HYAGNVDYN	I	gi|38347761
	536.77	1071.53	2	1071.53	L	GEQIDNLQR	V	gi|38347761
	375.19	1122.55	3	1122.56	S	YTQQVEDLK	R	gi|38347761
	586.28	1170.55	2	1170.54	R	NDNSSRFGKF	I	gi|38347761
	591.31	1180.60	2	1180.60	K	RQEAPPHIFS	I	gi|38347761
	593.31	1184.61	2	1184.61	E	LGEQIDNLQR	V	gi|38347761
	597.78	1193.54	2	1193.54	K	KKMEGDLNEM	E	gi|38347761
	408.22	1221.65	3	1221.66	K	KRQEAPPHIF	S	gi|38347761
	655.34	1308.67	2	1308.69	K	KRQEAPPHIFS	I	gi|38347761
	672.81	1343.61	2	1343.60	T	TNPYDYPFISQ	G	gi|38347761
	676.35	1350.69	2	1350.69	D	LQHRLDEAEQI	A	gi|38347761
Desmin	648.30	1294.58	2	1294.58	K	NVQEAEEWYK	S	gi|3676232
	691.82	1381.62	2	1381.62	K	NVQEAEEWYKS	K	gi|3676232
	755.86	1509.71	2	1509.71	K	NVQEAEEWYKSK	V	gi|3676232
alpha enolase-1	588.23	587.22	1	587.29	D	AINVGD	E	gi|11999261
	785.46	784.45	1	784.45	F	MILPIGAA	N	gi|11999261
	853.43	852.43	1	852.42	A	STGIYEAL	E	gi|11999261
	428.27	854.52	2	854.53	Q	VILPVPAF	N	gi|11999261
	485.29	968.57	2	968.57	Q	VILPVPAFN	V	gi|11999261
Creatine	526.76	1051.51	2	1051.51	T	GVDNPGHPFI	M	gi|392873934
Kinase	570.77	1139.53	2	1139.52	L	VWVNEEDHL	R	gi|392873935

a, Molecular ion mass observed in the nLC-MS/MS system in mass/charge (m/z); b, Expected molecular mass in Daltons calculated from the observed m/z; c, Charge of the ion; d, Calculated relative molecular mass in Daltons; f, Accession number of the protein in NCBInr database.

Table 2

Sequences identified using mass spectrometry in fraction F3 obtained from size-exclusion chromatography.

Protein Name	**Observe (*m/z*)**	**Expecte (Mr)**	**Charg $[M-H]^+$**	**Calculate (Mr)**	**P_0**	**Sequence**	**P_f**	**Acc.Numbe NCBInr**
Actin	519.25	518.24	1	518.25	V	WIGGS	I	gi|39287393

	272.16	542.31	2	542.34	A	IQAVL	S	gi|39287393
	303.15	604.29	2	604.30	G	IADRM	Q	gi|39287393
	625.29	624.29	1	624.29	L	FQPSF	I	gi|39287393
	644.29	643.28	1	643.29	P	IYEGY	A	gi|39287393
	646.35	645.34	1	645.35	G	IITNW	D	gi|39287393
	692.36	691.35	1	691.35	E	TLFQPS	F	gi|39287393
	347.17	692.32	2	692.34	K	IWHHT	F	gi|39287393
	349.22	696.43	2	696.44	S	IVGRPR	H	gi|39287393
	381.72	761.42	2	761.43	T	LKYPIE	H	gi|39287393
	395.22	788.43	2	788.44	R	AVFPSIVG	R	gi|39287393
	421.19	840.37	2	840.38	T	FNVPAMY	V	gi|39287393
	435.24	868.46	2	868.47	L	TEAPLNPK	A	gi|39287393
	436.75	871.48	2	871.49	V	FPSIVGRP	R	gi|39287393
	440.21	878.41	2	878.41	R	VAPEEHPT	L	gi|39287393
	460.21	918.41	2	918.42	G	FAGDDAPRA	V	gi|39287393
	469.18	936.35	2	936.36	T	NWDDMEK	I	gi|39287393
	477.73	953.44	2	953.45	H	NVPIYEGY	A	gi|39287393
	486.28	970.55	2	970.56	A	VFPSIVGRP	R	gi|39287393
	326.15	975.43	3	975.44	A	GFAGDDAPRA	V	gi|39287393
	488.72	975.43	2	975.44	K	AGFAGDDAPR	A	gi|39287393
	491.78	981.54	2	981.55	L	LTEAPLNPK	A	gi|39287393
	496.75	991.49	2	991.50	R	VAPEEHPTL	L	gi|39287393
	499.74	997.47	2	997.48	R	DLTDYLMK	I	gi|39287393
	518.26	1034.51	2	1034.51	L	RVAPEEHPT	L	gi|39287393
	518.83	1035.64	2	1035.64	K	IKIIAPPER	K	gi|39287393
	521.80	1041.59	2	1041.60	R	AVFPSIVGRP	R	gi|39287393
	534.25	1066.49	2	1066.49	D	AYVGDEAQSK	R	gi|39287393
	548.30	1094.59	2	1094.63	T	LLTEAPLNPK	A	gi|39287393
	574.81	1147.60	2	1147.60	L	RVAPEEHPTL	L	gi|39287393
	581.31	1160.60	2	1160.61	K	EITALAPSTMK	I	gi|39287393
	585.29	1168.57	2	1168.57	T	GIVLDSGDGVTH	N	gi|39287393
	391.89	1172.64	3	1172.66	K	IIAPPERKYS	V	gi|39287393
	591.76	1181.51	2	1181.52	K	DAYVGDEAQSK	R	gi|39287393
	400.23	1197.68	3	1197.70	R	AVFPSIVGRPR	H	gi|39287393
	618.77	1235.52	2	1235.53	T	NWDDMEKIW	H	gi|39287393
	420.52	1258.53	3	1258.54	N	WDDMEKIWH	H	gi|39287393
	632.79	1263.58	2	1263.58	G	IITNWDDMEK	I	gi|39287393
	661.31	1320.60	2	1320.60	H	GIITNWDDMEK	I	gi|39287393
	458.53	1372.57	3	1372.59	T	NWDDMEKIWH	H	gi|39287393
	694.88	1387.74	2	1387.75	G	IADRMQKEITAL	A	gi|39287393
	465.91	1394.71	3	1394.72	D	LAGRDLTDYLMK	I	gi|39287393
	349.90	1395.59	4	1395.60	N	WDDMEKIWHH	T	gi|39287393
	472.28	1413.83	3	1413.83	K	IKIIAPPERKYS	V	gi|39287393
	499.24	1494.68	3	1494.70	M	GQKDAYVGDEAQS	R	gi|39287393
	375.17	1496.63	4	1496.65	N	WDDMEKIWHHT	F	gi|39287393
	378.41	1509.62	4	1509.65	T	NWDDMEKIWHH	T	gi|39287393
	403.68	1610.67	4	1610.69	T	NWDDMEKIWHHT	F	gi|39287393
	575.60	1723.77	3	1723.78	I	ITNWDDMEKIWHH	T	gi|39287393
	460.22	1836.84	4	1836.86	G	IITNWDDMEKIWH	T	gi|39287393
	485.48	1937.89	4	1937.91	G	IITNWDDMEKIWH	F	gi|39287393
Myosin Heavy chain	404.19	403.18	1	403.17	F	AGADA	D	gi|38347761
	445.11	444.11	1	444.27	I	GALGK	A	gi|38347761
	260.12	518.24	2	518.29	Q	IAMKG	G	gi|38347761
	532.31	531.30	1	531.29	M	ASLEL	D	gi|38347761
	548.28	547.27	1	547.25	S	ADIET	Y	gi|38347761
	338.15	674.29	2	674.30	K	LYDQH	L	gi|38347761
	379.71	757.40	2	757.41	Q	IDNLQR	V	gi|38347761
	394.69	787.36	2	787.37	L	GEQIDNL	Q	gi|38347761
	413.23	824.44	2	824.45	K	RVIQYF	A	gi|38347761
	417.20	832.38	2	832.39	I	SERLEEA	G	gi|38347761
	435.20	868.39	2	868.40	Y	DYPFISQ	G	gi|38347761

	438.20	874.39	2	874.40	E	EEIEAER	A	gi|38347761
	472.27	942.53	2	942.54	R	LQDLVDKL	Q	gi|38347761
	498.24	994.46	2	994.47	K	YETDAIQR	T	gi|38347761
	526.72	1051.43	2	1051.44	G	HYAGNVDYN	I	gi|38347761
	536.77	1071.52	2	1071.53	L	GEQIDNLQR	V	gi|38347761
	562.29	1122.56	2	1122.47	T	PGTMDNNLVM	H	gi|38347761
	593.31	1184.60	2	1184.61	E	LGEQIDNLQR	V	gi|38347761
	595.32	1188.62	2	1188.62	L	KKDIDDLELT	L	gi|38347761
	617.28	1232.54	2	1232.54	Q	VDDLEGSLEQE	K	gi|38347761
	651.83	1301.64	2	1301.64	K	IAEKDEEIDQI	K	gi|38347761
	357.43	1425.69	4	1425.71	T	VRNDNSSRFGKF	I	gi|38347761
Type I	428.24	427.24	1	427.24	G	IAGPA	G	gi|65736617
alpha 1 chain	445.11	444.11	1	444.23	A	GAIGQ	R	gi|65736617
	223.10	444.19	2	444.24	G	ARGAA	G	gi|65736617
	229.15	456.29	2	456.24	K	AGRPG	D	gi|65736617
	229.15	456.29	2	456.24	E	GAPGR	D	gi|65736617
	472.25	471.24	1	471.23	G	DIGAP	G	gi|65736617
	238.15	474.29	2	474.21	P	AGPSGS	S	gi|65736617
	238.16	474.30	2	474.21	G	QSGPS	G	gi|65736617
	239.13	476.25	2	476.22	G	KDGAS	G	gi|65736617
	488.28	487.27	1	487.30	L	LLAAT	L	gi|65736617
	246.24	490.46	2	490.24	G	SEGAK	G	gi|65736617
	517.24	516.23	1	516.22	G	SPGAEG	A	gi|65736617
	266.17	530.32	2	530.27	G	PKGET	G	gi|65736617
	542.26	541.25	1	541.31	E	RGRPG	A	gi|65736617
	547.28	546.28	1	546.26	G	EQGIT	G	gi|65736617
	573.30	572.29	1	572.28	R	TGEIGP	V	gi|65736617
	578.27	577.27	1	577.29	D	GPPGPPG	L	gi|65736617
	295.11	588.20	2	588.27	G	MPGER	G	gi|65736617
	602.22	601.21	1	601.28	G	ERGSPG	P	gi|65736617
	614.30	613.29	1	613.32	G	DRGLPG	L	gi|65736617
	679.28	678.28	1	678.28	S	HGGFDF	Q	gi|65736617
	709.39	708.38	1	708.38	G	IAGPPGPT	G	gi|65736617
	428.20	854.39	2	854.41	A	GPSGPMGPR	G	gi|65736617
	540.76	1079.51	2	1079.52	A	GPAGPSGPMGPR	G	gi|65736617
	611.80	1221.59	2	1221.59	G	AAGPAGPSGPMGP	G	gi|65736617
	665.30	1328.59	2	1328.59	K	TSGGIPMPGPMGP	G	gi|65736617
Fast tropomyosin	716.86	1431.71	2	1431.71	L	VIIESDLERTEE	R	gi|13784996
	736.87	1471.72	2	1471.73	K	LDKENALDRAEQA	E	gi|13784996
GAPDH	538.26	537.25	1	537.25	E	GLMTT	V	gi|88604792
	442.18	882.34	2	882.35	K	WGDSGAQY	V	gi|88604792
	659.84	1317.66	2	1317.66	R	VIISAPSADAPMF	V	gi|88604792
	737.89	1473.76	2	1473.77	K	RVIISAPSADAPMF	V	gi|88604792
Enolase 3	488.28	487.27	1	487.30	I	LGVSL	A	gi|11999263
	428.27	854.52	2	854.53	D	VILPVPAF	N	gi|11999263
	485.29	968.56	2	968.57	D	VILPVPAFN	V	gi|11999263

a, Molecular ion mass observed in the nLC-MS/MS system in mass/charge (m/z); b, Expected molecular mass in Daltons calculated from the observed m/z; c, Charge of the ion; d, Calculated relative molecular mass in Daltons; f, accession number of the protein in NCBInr database.

Table 3. ACE inhibitory activity (IC_{50}) of synthetic peptides.

Fraction	Peptide	IC_{50} (μM)
2	ESAGIH	371.60
	TLKYP	170.00
	LKYPI	27.07
	IVGRPR	>1000
3	FQPSF	12.56
	IYEGY	79.42
	IITNW	30.96
	IVGRPR	>1000

Figure 1.
Degree of hydrolysis curve (A). ACE inhibitory activity of TRMH-A26 at different concentrations (B).

Figure 2.

Elution profile of TRMH-A26 obtained after SEC (A); and IC_{50} of ACE inhibitory activity of fractions (B).

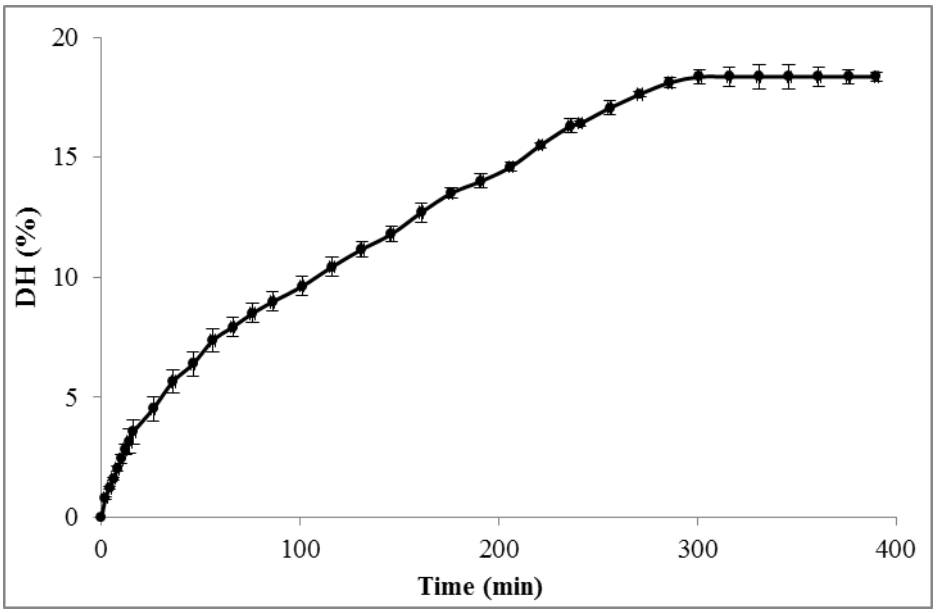
Figure 3.

A and B) Distribution of the identified peptides in fraction F2 and F3, respectively.

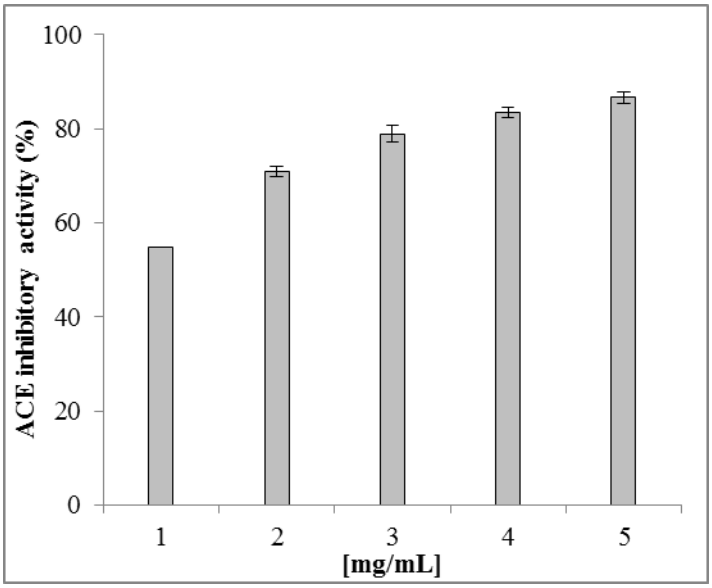
401

402 **Figure 1.**

A)



B)



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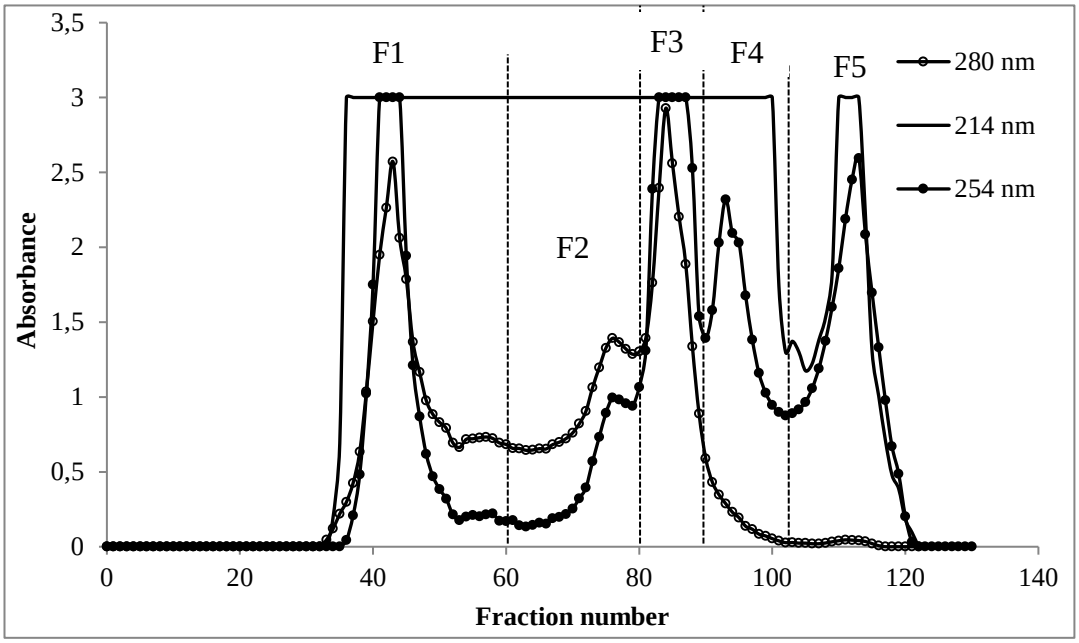
406

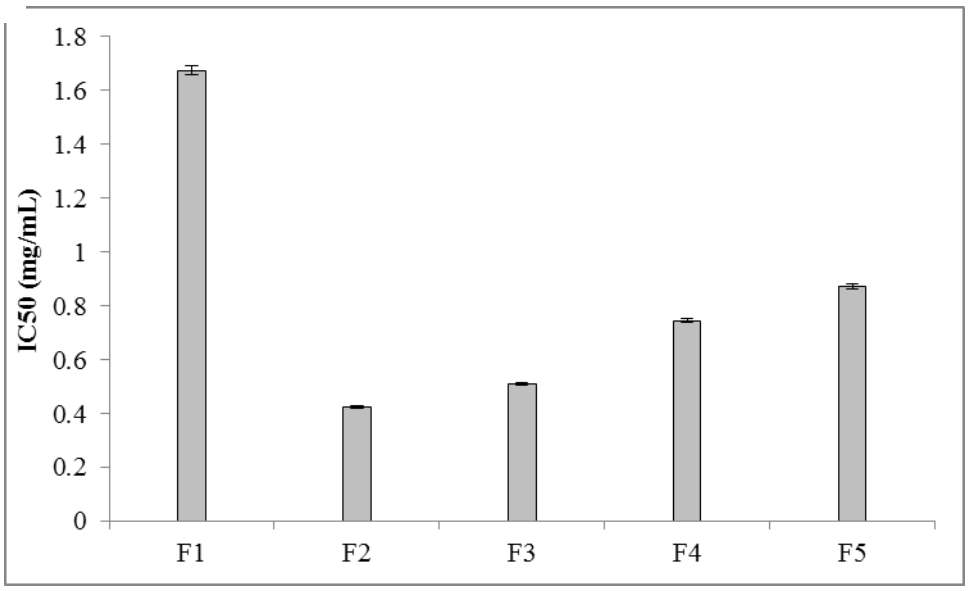
407

Figure 2.

A)


3,5
3
2,5
2
1,5
1
0,5
0
Absorbance
0
20
40
60
80
100
120
140
Fraction number
F1
F2
F3
F4
F5
280 nm
214 nm
254 nm

B)

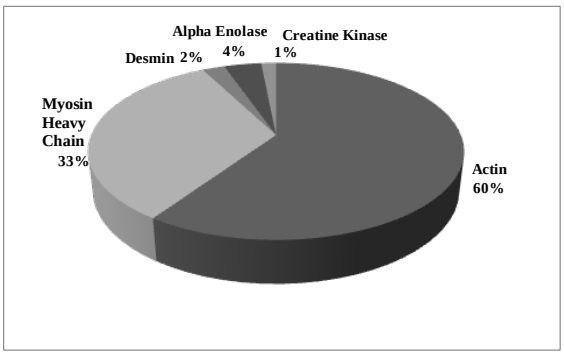

1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
IC50 (mg/mL)
F1
F2
F3
F4
F5

422

423 **Figure 3.**

424

42 **A**
)



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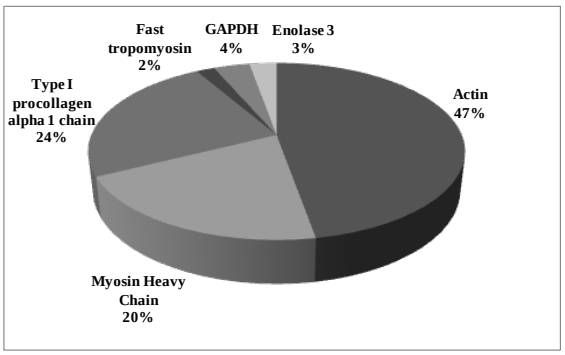

426

427