Preparation and identification novel angiotensin-converting enzyme-inhibitory peptides derived from fish waste

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Abstract. The purpose of the research is to compare short peptides from different hydrolysates of fish entrails that can inhibit the activity of angiotensin-converting enzyme (ACE). Fish hydrolysates derived from Catfish, Tilapia, and Mackerel entrails were digested by pepsin and passed through the 3kDa cutoff column. The fraction containing peptides shorter than and equal to 3kDa from catfish hydrolysate has the great ability to inhibit ACE activity in converting the substrate (Furanacroloy-Phe-Gly-Gly, FAPGG) and producing FAP and GG as the products of the reaction. The 3kDa filtrate from the catfish hydrolysate had activity like Captopril, a drug for treating hypertension. The 3kDa filtrate derived from the catfish entrail hydrolysate was purified using OFFGEL electrophoresis and then passed through the C18 column. The 3kDa filtrate was separated into two fractions and then these fractions were determined ACE inhibitory activity. The result showed that the fractions containing hydrophilic peptides and others containing hydrophobic peptides possessed inhibitory activity against ACE. Those fractions were analyzed with LC-MS/MS for sequencing. The results revealed that synthesized peptides; ASNLHGV, LFKDLR, PGYALQR, and LETAKSR, derived from the catfish hydrolysate showed anti-ACE activity against its substrate.

1. Introduction

Operations in fish processing (washing, degutting, salting, fermentation, drying, and smoking) has yielded more than 60% by-products [1]. Waste from fish processing, especially in washing and degutting cause disposal problem and increase an expense for management to seafood industries. By-products of fish, such as head, bones, frames, tails, skin, and viscera remain a huge of protein sources and high level of essential micronutrients such as vitamin A, D, B12, and minerals such as calcium, phosphorus, iron, zinc, selenium, and iodine [2]. A process to manage fish by-products as the source of protein has added value of fish wastes within industrial sections.

Hypertension causes several health problems and is the most risk factor of heart disease, stroke, as well as sometimes death. More than 9.4 million deaths worldwide every year was a few deaths reasoned from complications of hypertension [3]. Angiotensin-converting enzyme (ACE) inhibitors are an option of medications for hypertension. Angiotensin-converting enzyme (ACE) is an enzyme involving blood pressure regulated by the renin-angiotensin system. This enzyme converts biologically inactive angiotensin I to the potent vasoconstrictor angiotensin II that is a key factor for an increase in the arterial pressure. The renin-angiotensin system has become an important target for developing antihypertensive drugs[4]. Captopril and enalapril are synthetic ACE inhibitors used to prevent hypertension in clinical [5]. The negative side effects from these synthetic ACE inhibitors such as skin rashes, allergic reactions, cough, headache, and renal impairment have been concerned from utilizations [6]. An approach to prevent these side effects needs to figure out safer, more innovative, and economical ACE inhibitor for the treatment of hypertension in human. The replacement of synthetic ACE inhibitors by natural substances, for instance ACE-inhibitory peptides can have benefit as the result of health implications.

Bioactive peptides (BPs) are organic compounds made from amino acid linked by covalent bonds. They has effects on the digestive, endocrine, cardiovascular, immune and nervous systems of human [7]. Mostly, BPs have a size between 2-20 residues, and usually have a molecular weight <3 kDa. Moreover, they contain hydrophobic amino acids besides proline, lysine or arginine group [8]. Based on their functional properties, BPs are classified as anti-microbial, anti-thrombotic, antihypertensive, opioid, immunomodulatory, mineral binding, and antioxidant actions [9, 10]. Even though BPs have been isolated and characterized from several natural sources, Fish are the relevant source of many BPs. The identification and characterization of biologically active peptides hydrolyzed form numeral kinds of fish have been achieved, and the results reveal their potential actions in antihypertensive, antioxidative, antimicrobial, and antiproliferative effects [11-14].

Recently, several studies are interested in the production and isolation of ACE-inhibitory peptides from fish waste hydrolysates of fish industries and cuttlefish wastewater [15, 16]. However, the inhibitory hypertensive activity of low molecular weight peptides obtained from fish entrails has not been elucidated and reported. In this work, we described the peptide sequences and their antihypertensive of fish entrail waste peptides. identification of new, nontoxic, safe, and inexpensive ACE inhibitors from fish wastes is aim of this study.

2. Materials and Methods

2.1 Materials

Fish entrails separated from catfish, tilapia and mackerel were collected at a local market of Samutprakarn province, Thailand. Pepsin (EC 232-629-3, 3,000 NFU/mg) was purchased from Himedia (Mumbai, India). Amicon® Ultra-15 Centrifugal Filter 3kDa cut off column and purified columns were purchased from Merk (Darmstadt, Germany). N-[3-(2-Furyl) acryloyl]-L-phenylalanyl-glycyl-glycine, FAPGG, Angiotensin Converting Enzyme from rabbit lung, ACE [≥2.0 units/mg protein (modified Warburg-Christian)], and Captopril were purchased from Sigma Chemical Co. (St. Louis, USA). All other reagents were analytical reagent grade.

2.2 Preparation of fish entrail hydrolysates

Fish entrails of catfish, tilapia and mackerel were soaked with distilled water and 95% ethanol to remove lipid. The mixture was centrifuged at 3000x g for 10 mins. The supernatant was removed, and the crude entrails were dried in an incubator at 80 ºC. The dried samples were kept in plastic bags and stored at -20 °C until use. The crude entrails were dissolved in 50mM sodium acetate buffer, pH 3.7 (1:5 w/v) and digested by pepsin at 37 ºC for 16 hours with an enzyme/the crude entrails ratio of 1:25. The hydrolysate course was finished by heating at 90 ºC for 10 mins. The supernatant from the hydrolysate was collected by centrifugation at 10,000 x g for 10 mins. The Protein concentration was determined by Bradford method [17] with bovine serum albumin (BSA) utilized as the standard.

2.3 ACE-inhibitory activity assay

ACE-inhibitory activity was measured according to the previous published procedure [18] with modification. The substrate, N-[3-(2-Furyl) acryloyl]-L-phenylalanyl-glycyl-glycine, FAPGG, was buffered with 50 mM HEPES buffer containing 0.3 M and 10 µM $ZnCl_2$ pH 7.5. The reaction was mixed in 1.5 mL Eppendorf tube by adding 10 µL of enzyme solution and 10 µL of 5 mM FAPGG. Captopril, an angiotensin-converting enzyme (ACE) inhibitor, was used as a reaction of control. The reactions were incubated in 37ºC heat box for 5 min. Decreasing of an absorbance at 334 nm was monitored every 1 min for a period of 20 min. Each activity value was determined from an average of three replicates.

2.4 Fraction and purification of fish protein hydrolysate

The protein hydrolysate was fractionated with the 3 kDa Centrifugal Filter cut off spined column. The fractions containing below 3 kDa peptides were collected, and ACE inhibitory activities of these fractions were determined. The below 3 kDa fractions were purified with OFFGEL fractionator to separate each peptide depended on their isoelectric point (pl) of peptides. The different samples were collected in an IPG (Immobilized pH gradient) gel strip used in the fractionator. Then, the samples obtaining ACE activity were purified with reversed-phase C18 column to determine hydrophobic peptides or hydrophilic peptides with ACE inhibitory activities.

2.5 Nano/Capillary LC-MS/MS analysis

The hydrolysate with 0.1% formic acid was subjected to Nano/Capillary LC (Ultimate3000 LC System, Thermo Scientific, UK) for peptide separation in C18 column (75 µm X 15 cm) (Acclaim PepMap RSLC, Thermo Scientific). This instrument was designed to operate with Hybrid quadrupole Q-ToF impact II™ (Bruker Daltonics), and Nano-captive spray ion source. The elution was performed in linear gradient mode from 0.1% formic acid to 80% acetonitrile containing 0.1% formic acid for 30 mins at a flow rate of 0.30 µL/min. The peptide sequencing was identified in the fraction that revealed remarkable ACE-inhibitory activity by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF-TOF MS). The MS/MS data was analyzed with Bruker compass data analysis 4.4 software (Bruker Daltonics). The results were represented in mz X ML format of CompassXport 3.0 software (Bruker Daltonics). The peptide sequencing was analyzed using DeCyder MS differential analysis 2.0 software (DeCyderMS, GE Healthcare) and Mascot software (Matrix Science). The peptides were matched to the published sequence of the relative protein data in Scombridae database (NCBInr databank).

2.6 Peptide synthesis

The peptides with potential ACE-inhibitory activity determined by possibility of amino acids in peptide sequences were chemically synthesized by GenScript Biotech (NJ, USA). The purity (all above 94%) and sequence of those peptides were verified by HPLC. ACE inhibitory activities of the chemically synthesized peptides were determined as described in the method above.

3. Results and Discussion

3.1 Preparation of fish entrail waste protein hydrolysate

In the experiment, dried entrails derived from catfish, tilapia, and mackerel were buffered in the 50 mM sodium acetate pH 3.7 that is a condition for proteolytically active pepsin found in the gastric juice of all vertebrates [19]. Pepsin hydrolysate of all dried fish entrails of catfish, tilapia, and mackerel showed 0.921, 0.640, and 1.001 mg/mL of protein concentration, respectively. Fish hydrolysates were fractionated in ultrafiltration membrane of molecular weight cut off (MWCO) for enrichment of fish hydrolysates in low molecular weight ACE-inhibitory peptides (< 3kDa). Obtained protein concentrations in the <3kDa hydrolysates were 0.381 mg/mL, 0.529 mg/mL, and 0.189 mg/mL found in the hydrolysates of catfish,

tilapia, and mackerel, respectively. 30 g of dried entrail waste of tilapia provided the highest < 3 kDa protein, although initiation in protein concentration before the 3kDa cut-off fractionation was not the highest of those.

3.2 ACE-Inhibitory Determination of fish hydrolysates in low molecular weight ACE-inhibitory peptides (< 3kDa)

An optimal concentration between substrate and ACE extracted from rabbit lung (sigma, USA) was required for ACE-inhibitory test. Substrate N-[3-(2-Furyl) acryloyl]-L-phenylalanyl-glycyl-glycine, FAPGG (sigma F7173, USA) was varied in the range of 0.1- 20 mM of substrate concentration to determine the optimal concentration for further experiment. The enzymatic activity was monitored by decrease in absorbance at 340 nm as the result of FAPGG hydrolysis to FAP and GG [20]. Figure 1 shows the relationship between the decrease in absorbance at 334 nm and time in hydrolysis of different FAPGG concentrations of 0.1, 0.5, 1, 3, 5, 10, 15, and 20 mM as depicted in A-H, respectively. The negative value of a slope of each graph represented the ACE activity. The negative slopes of FAPGG concentration between 3-20 mM were not significantly different. 5 mM of FAPGG was a candidate concentration to use for ACE-inhibitory assay. To optimize rabbit ACE concentration, 0.1, 0.5, and 1 Units of ACE were used to hydrolyze 5 mM FAPGG. The results showed -0.27, -2.00, and -2.26 that were values of negative graph slopes indicated for using 0.1, 0.5, and 1 Units of ACE, respectively (Figure 2). The slope values shown in the graphs of using 0.5 and 1 Units of ACE concentrations were not significantly different. Therefore, 0.5 Unit of enzyme was enough for hydrolysis assay.

Fish entrail hydrolysates obtained after enzyme hydrolysis and 3kDa fractionations were tested for ACE inhibitory activity. The ACE inhibitory activity was determined from an increase in absorbance at 334 nm, the specific wavelength of the substrate. The results exhibited that all <3kDa fractions derived from catfish, tilapia, and mackerel hydrolysates showed the ACE inhibitory activity. Table 1 shows the comparative results between the reactions with and without inhibitors. The negative slope was interpreted as the reaction without the inhibitor (negative control), whereas the mixture with the inhibitor showed an increase slope value from the negative control. Captopril was used as a commercial ACE inhibitor in the experiment. There are increases of graph slopes in the reactions containing <3kDa fractions (Table 1). The activity was determined at 5, 10, 15, and 20 mins of reaction time. Velocity in the decrease of the substrate per time of angiotensin converting enzyme inhibitors (ACEIs) showed the maximum at 5 mins. At this time, the <3kDa fraction extracted from catfish showed the highest ACE inhibitory activity and the result was related with the result using captopril (Figure 3).

3.3 Identification of the ACE inhibitory peptide sequences from fish entrail hydrolysates

The <3kDa hydrolysate extracted from catfish was purified to separate peptides following an isoelectric point (PI) of each peptide. The hydrolysate was applied in OFFGEL Electrophoresis, and the samples were collected in the range of pH 3-10. The results showed ACE inhibitory activity in peptides separated in pH 5-6 and 8-9. All fractions obtained ACE inhibitory activity were applied in C_{18} column to determine the activity between hydrophilic and hydrophobic peptides. Several studies have been indicated that hydrophobic amino acid residues composed of aliphatic side chains, for example Glycine (G), Alanine (A), Valine (V), Leucine (L), and Isoleucine (I) at the C-terminal of peptides have affected on increase of the ACE inhibitory activity [21-23]. Table 2 shows the increase of graph slopes in all samples, except the sample obtained from the hydrophilic fraction pH 5. It replied that ACE inhibitory activities were found in both hydrophobic fraction's pH 5, 6, 8, 9 and in hydrophilic fraction 's pH 6, 8, 9. The hydrophobic fraction pH 9 showed the highest ACE inhibitory activity and revealed an inhibitory activity the same as using captopril.

The sequence of the peptides in both hydrophobic fraction's pH 5-9 and in hydrophilic fraction's pH 6-9 were investigated by the method of Nano/Capillary LC-MALDI-TOF-MS/MS. The 15 peptide sequences were identified as AVLGSLGSAGGPKK, SVLGGSGPKPQLR, LGSAVLGSAGPKGK, LFKDLR, LFKFVR, GPYALQR, PGYALQR, KFAGPSVLAPPPL, KFAGPLPAVSPPL, KFAGPVSLAPPPL, RTPAAPKPGLLTLLVPF, ASNVVHA, ASNLHGV, ASNYSR, LETAKSR. The amino acid within a peptide sequence has a key effect on ACE inhibitory activity. The positively charged amino acids (L and R) have been associated with the stronger efficiency of ACE inhibitory peptides. Additionally, hydrophilic amino acids in peptides provide weak or no ACE inhibition [24, 25]. LFKFVR, KFAGPSVLAPPPL, ASNLHGV, LFKDLR, PGYALQR, and LETAKSR were chemically synthesized as potent peptide sequences. Except LFKFVR, other peptide sequences presented an ACE inhibitory activity (Table 3). Peptides with sequences PGYALQR and LETAKSR obtained higher ACE inhibitory activity than captopril. ACE inhibitory activity should be tested in vivo for their potential use in the treatment of diseases involving hypotension. However, there are invivo and clinical studies of marine-sourced ACE-inhibitory peptides in spontaneously hypotensive rat, such as LEPWR from muscle of mackerel pike; LSGYGP from muscle of Nile tilapia; PPLLFAAL from muscle of yellowbelly pufferfish; VELYP from muscle of cuttlefish; GWA from muscle of pearl

oyster showed their potential to lower blood pressure over time [26-28]

4. Conclusions

The renin-angiotensin-aldosterone system (RAAS) is important in the human metabolisms to maintain the organism's homeostasis, control of fluid and electrolyte balance, and regulate in response system to physiological and pathological conditions [29]. The ACE is the most relevant enzyme in this system due to its function to produce of high concentrations of angiotensin II that contributes hypertension, atherosclerosis, thoracic aortic aneurysms, or abdominal aortic aneurysms [30]. Many ACE inhibitors have been developed. Captopril is the most common ACE inhibitor used as a drug to reduce hypertension in a patient. However, adverse effects of captopril are skin rash and loss of taste [31]. ACE inhibitory peptides are the potential alternative to replace a treatment with chemical drug. The results in this research show that the potent peptides derived from fish entrails have potential to be an ideal drug for clinical therapies.

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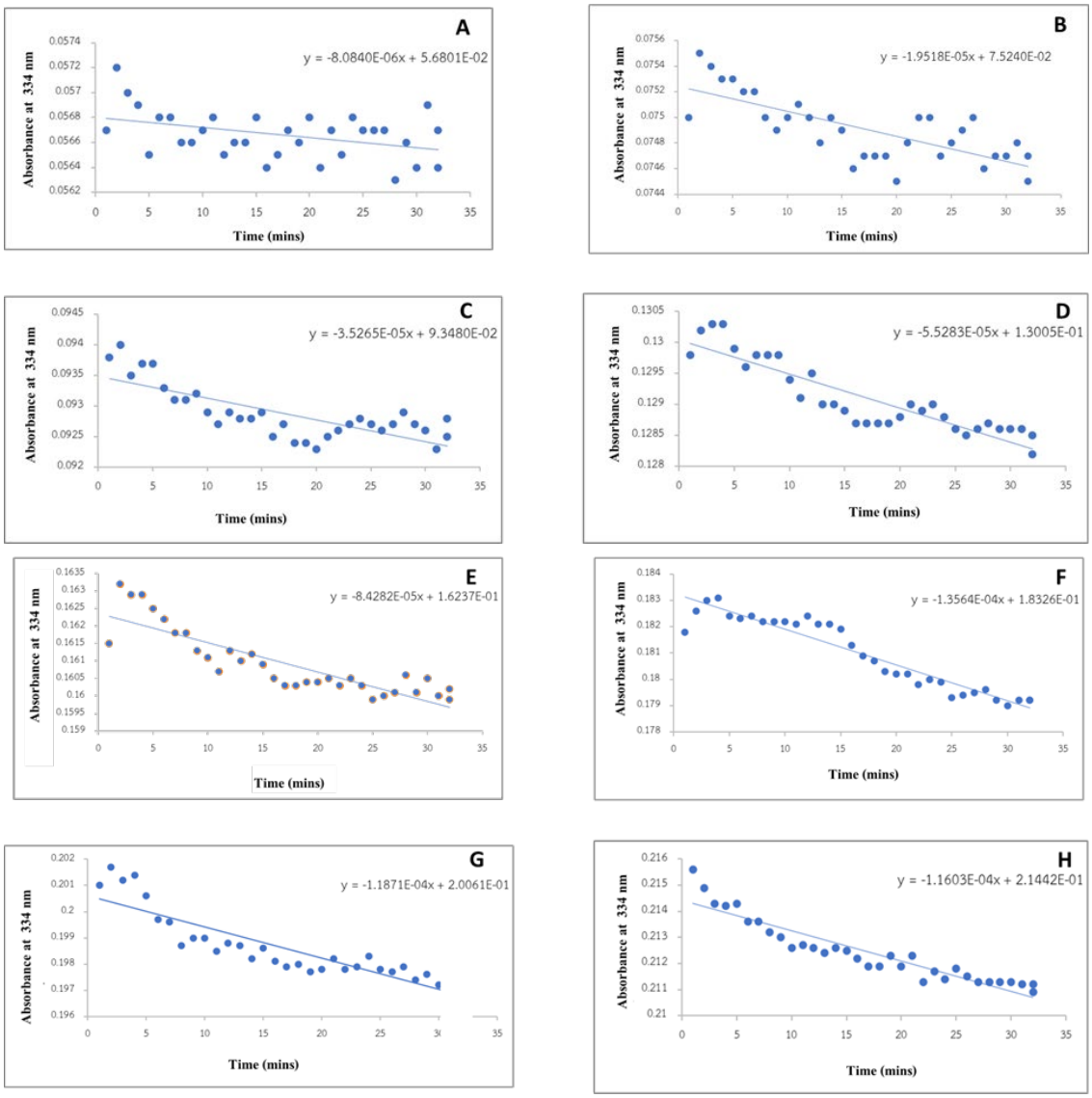

Figure 1. The relationship between decrease in absorbance at 334 nm and time. The negative slope values of -0.08, -0.02, -0.35, -0.55, -0.84, -1.36, -1.19, and -1.16 represented an enzyme activity against substrate concentrations of 0.1, 0.5, 1, 3, 5, 10, 15, and 20 mM of FAPGG, respectively as shown in the graph (A-H).

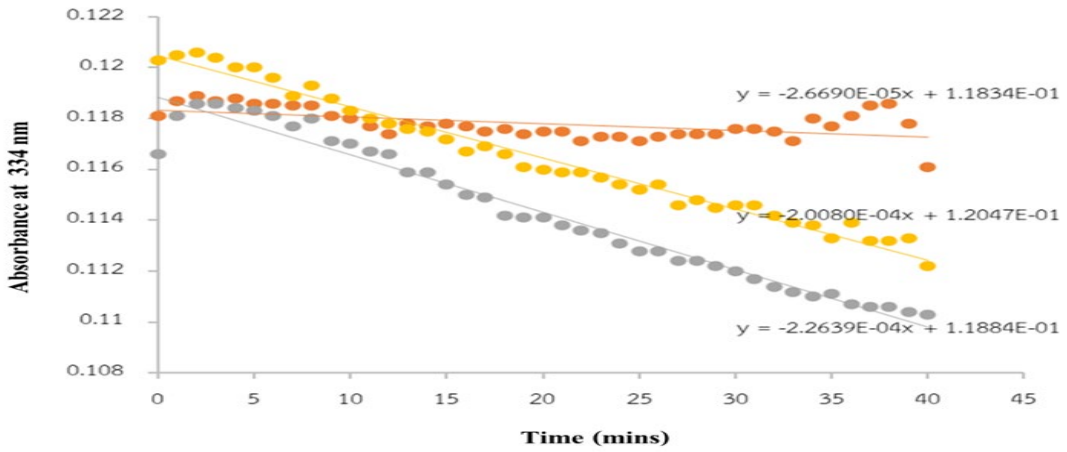

Figure 2. The comparative graph represented FAPGG hydrolysis in different ACE concentrations. The orange dots showed the line graph of 0.1 mU of ACE, the yellow dots indicated the line graph of 0.5 mUof ACE, and the gray dots revealed the line graph of 1 mU of ACE

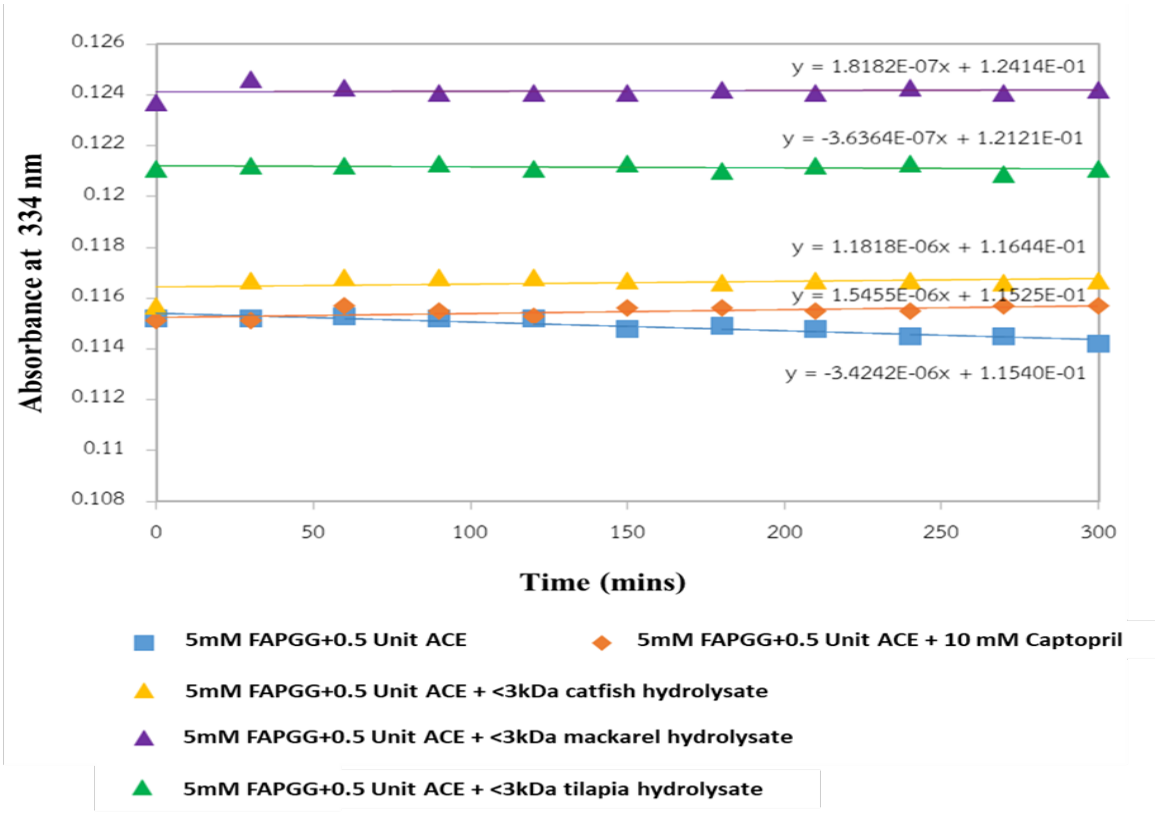

Figure 3. ACE inhibitory activity in <3kDa fish hydrolysate from different fish entrails. The inhibitory activity was interpreted from the increase of graph slope over time.

Table 1. Comparative results of ACE inhibitory activity in <3kDa fish hydrolysate determined from velocity of the substrate hydrolysis.

Samples	Graph slopes of FAPGG hydrolysis over time			
	5 mins	10 mins	15 mins	20 mins
Velocity of substrate hydrolysis over time (using captopril)	4.97	4.16	4.44	4.48
ACE from rabbit lung, (sigma, USA) + FAPGG	-3.42	-3.84	-4	-3.96
ACE from rabbit lung, (sigma, USA) + FAPGG + Captopril	1.55	0.32	0.44	0.52
ACE from rabbit lung, (sigma, USA) + FAPGG + <3kDa catfish hydrolysate	1.18	-0.45	-0.94	-1.23
ACE from rabbit lung, (sigma, USA) + FAPGG + <3kDa tilapia hydrolysate	-0.36	-0.06	0.23	0.29
ACE from rabbit lung, (sigma, USA) + FAPGG + <3kDa mackarel hydrolysate	0.18	0.13	0.55	0.87

Table 2. ACE inhibitory activity of hydrophobic and hydrophilic peptides derived from catfish entrail hydrolysate. The activity was determined from velocity of the substrate hydrolysis and described as slope values.

Samples	A graph slope of FAPGG hydrolysis over time
ACE + FAPGG	-1.4569
ACE + FAPGG + captopril	0.0956
ACE + FAPGG + hydrophilic fraction pH 5	-4.5210
ACE + FAPGG + hydrophobic fraction pH 5	-0.6427
ACE + FAPGG + hydrophilic fraction pH 6	0.0047
ACE + FAPGG + hydrophobic fraction pH 6	-0.2661
ACE + FAPGG + hydrophilic fraction pH 8	-1.1237
ACE + FAPGG + hydrophobic fraction pH 8	-0.8322
ACE + FAPGG + hydrophilic fraction pH 9	-0.7929
ACE + FAPGG + hydrophobic fractions pH 9	0.1110

Table 3. ACE inhibitory activity of chemically synthesized peptides. The activity of each peptide was determined from velocity of the substrate hydrolysis and described as slope values.

Samples	A graph slope of FAPGG hydrolysis over time
ACE + FAPGG	-2.2711
ACE + FAPGG + captopril	1.067
ACE + FAPGG + LFKFVR	-2.9979
ACE + FAPGG + KFAGPSVLAPPPL	-1.2329
ACE + FAPGG + ASNLHGV	0.8901
ACE + FAPGG + LFKDLR	0.3963
ACE + FAPGG + PGYALQR	2.2313
ACE + FAPGG + LETAKSR	10.028