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Review article

PURIFICATION AND CHARACTERIZATION OF ACE INHIBITORY PEPTIDE FROM AQUATIC RESOURCES: A REVIEW

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ABSTRACT: Protein purification is an essential first step for the study of its molecular and biological properties in order to understand its specific biological function. There are several properties such as molecular weight, charge, hydrophobicity, amino acid composition, and etc. that can be exploited to purify or single out a target peptide from a mixture. Base on those properties, several chromatographic and non-chromatographic procedures have become available. The obtained protein hydrolysate from hydrolysis reaction showing inhibitory activity against ACE purified by using different techniques such as non-chromatographic (ultrafiltration; UF), and chromatographic (gel filtration; GF, ion exchange; IE, or reversed phase high performance liquid chromatography; RP-HPLC). After purification step, the potent fraction is indispensable tools for peptide mapping and protein primary structure elucidation by using mass spectrometry (MS) techniques, HPLC separation or protein sequencing for detecting molecular mass, amino acid sequence or amino acid composition. This article is presented the purification and characterization of ACE inhibitory peptides obtained by enzymatic hydrolysis of proteins from aquatic resources. Identification techniques of the peptides are also reviewed. The methods of purification and identification are significant steps that increase purity and efficacy of potent ACE inhibitory peptide (ACEIP).

Keywords: aquatic resources; purification; protein hydrolysate; active peptides; angiotensin converting enzyme, inhibitor

INTRODUCTION

After enzymatic hydrolysis, protein hydrolysate was determined for bioactivity including angiotensin converting enzyme (ACE) inhibitory activity. The ACE inhibitory activity of derived hydrolysate is usually analyzed *in vitro* and expressed in terms of IC_{50}, defined as the sample required inhibiting 50% of the ACE [1]. The determination of ACE inhibitory activity was detected by means of synthetic substrates with amino-substituted tri and dipeptides, such as hippuryl-L-histidyl-L-leucine (HHL) [1] and 2-furanacryloyl-L-phenylalanyl-L-glycyl-L-glycine (FAPGG) [2-4], via radioisotopic, spectrophotometric, fluorometric and chromatographic methods [4]. The other methods are reverse-phase high-performance liquid chromatography (RP-HPLC) used for screening purposes only, in order to discard inactive fractions obtained after the second fractionation [5]. After potent hydrolysates obtained, they were then fractionated based on peptide size/molecular weight, which is typically performed by using ultrafiltration [6-10]. The hydrolysate fraction displaying the highest bioactivity was then further purified by using different techniques for instance gel filtration/permeation [11-13], ion exchange/fast protein liquid chromatography (FPLC) [14-16] and RP-HPLC [17-21]. The combined techniques of mass spectrometry and protein sequencing are performed for detecting molar mass and amino acid sequence/composition in the potent ACE inhibitory peptide (ACEIP) [10-13, 22].

Purification of the potent hydrolysate is the critical first step for the study of its molecular weight and biological properties in order to understand its biological function. There are several properties such as molecular weight, charge, hydrophobicity, etc. that can be exploited to purify or single out a target peptide from a mixture. The protein hydrolysate showing inhibitory activity against ACE were then further purified by using different techniques such as non-chromatographic (ultrafiltration; UF), and chromatographic (gel filtration; GF, ion exchange; IE, or RP-HPLC) [23]. After purification step, the potent fraction is indispensable tools for peptide mapping and protein primary structure elucidation by using MS techniques, HPLC separation or protein sequencing for detecting molecular mass, amino acid sequence or amino acid composition [5].

After enzymatic hydrolysis, purification and identification have been already applied to liberate and yield the best potent peptides, the potent peptides exhibited various biofunctional activities such as antihypertensive [24-26], antioxidative [10, 27, 28], antimicrobial [29], cholesterol-lowering [30, 31], anticancer [32, 33], cellular oxidation stress inhibition [34], antithrombotic, mineral absorption enhancement, and immunomodulatory properties [35, 36]. To obtain the bioactive peptides with high efficacy, it needs to take purification and identification step in consideration. This article presented the purification and characterization of ACE inhibitory peptides obtained by enzymatic hydrolysis of proteins from aquatic resources. Identification techniques of the peptides are also reviewed.

Methods for ACE inhibitory activity determination

Numerous *in vitro* techniques, base on either spectrophotometic or HPLC assay, have been developed for the detection of ACE inhibitory activity of bioactive peptides. *In vitro* ACE-inhibitory activity is generally measured by monitoring the conversion of an appropriate substrate by ACE in the presence and absence of inhibitors [37]. For spectrophotometic assay, method of Cushman et al. [1] was broadly used [15, 31, 38-43]. The method based on the hydrolysis of HHL by ACE to Hippuric acid (HA) and His-Leu, and the extent of HA released is measured after its extraction with ethyl acetate (Figure 1). For this assay, ACE inhibitory activity is measured through the absorbance of HA after the reaction of hydrolysate samples on HHL [1]. The quantity of HA produced by ACE is measured spectrophotometrically at 228 nm using a UV-visible spectrophotometer. Apart from, HHL, the FAPGG [9, 11, 44-47] has been employed as substrates. Another broadly used spectrophotometric method is based on the hydrolysis of FAPGG to furylacryloylphenylalanine (FAP) and glycylglycine (GG). The hydrolysis of FAPGG by ACE will result in a decrease in absorbance at 340 nm [44, 45]. ACE inhibitory activity was calculated according to Equation (1).

$$\text{ACE inhibition (\%)} = \{(B-A)/(B-C)\} \times 100 \qquad (1)$$

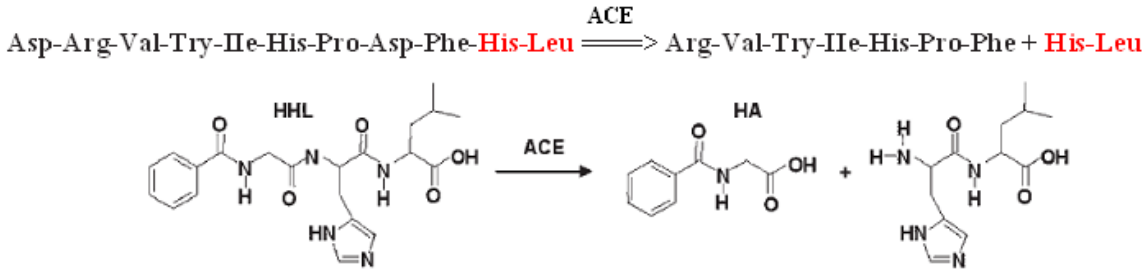

Figure 1. Analyzed reactions of angiotentin converting enzyme. Top: Cleavage of the natural substrate angiotensin I leading to angiotensin II. Bottom: The conversion of the artificial substrate hippuryl-L-histidyl-L-leucine (HHL) into its product hippuric acid (HA) Siemerink et al [37]

where A is the absorbance of HA produced in the presence of ACE, substrate and ACE inhibitor component; B is the absorbance of HA produced in the presence of ACE and substrate without ACE inhibitor component, and C is the absorbance of HA produced in the presence of substrate without ACE and ACE inhibitor component. IC_{50} value was defined as the concentration of hydrolysate (mg/ml) which inhibited ACE activity by 50% [39].

RP-HPLC is another method used for screening purposes only, in order to discard inactive fractions obtained after the second fractionation [5]. However, HPLC methods show lower detection sensitivity, but longer analysis times are required to obtain good results [48].In this case, the released HA was quantified by RP-HPLC on C-18 column. The absorbance is monitored at 228 nm and percentage of ACE inhibition can be calculated as peak area with HA liberated by ACE. The IC_{50} value was defined as the concentration of inhibitor required to reduce the HA peak by 50% (indicating 50% inhibition of ACE) and calculated by using the same equation in spectrophotometric assay [10, 13, 18, 49].

Purification of ACE inhibitory peptides (ACEIP)

After enzymatic hydrolysis, the purification of ACE inhibitory peptides is an essential first step for the study of its molecular and biological properties. After determination for ACE inhibitory activity of potent hydrolysate, the potent peptides that showed the highest ACE inhibitory activity need to further purification. Purification of ACEIP has been investigated by using single or consecutive several techniques including UF [6-10], GF [11-13], IE [14-16], and HPLC [17-21]. The summarized procedure for purification and identification of bioactive peptides from aquatic resources was depicted in Figure 2. The purification techniques normally used to increase purity of the peptides were summarized in Table 1. Moreover, the methods, type of membrane and column, and the conditions used for ACEIP production were also summarized in Table 2.

Ultra filtration (UF)

UF is a technique used commonly both on laboratory and commercial scale to fractionate, purify and concentrate proteins [23, 50]. Although it is possible to obtain very pure protein fractions using suitable columns as in column chromatography, UF is a low cost and easy to scale-up method used widely in food industries for quick separation and concentration of food proteins [9]. Many researchers selected UF method in the first fractionation of ACEIP [9-11, 51, 53] because a wide variety of large, medium and small peptides are generated depending on the enzyme specificity and the extent of hydrolysis during the hydrolysis reaction. From Table 2, different molecular weight cut off (MWCO) membranes ranged from 1000 to 5000 Da were used in fractionation of protein hydrolysate from sea cucumber [7, 8], Loach muscle [11], Pangasius catfish skin and bone [51], and squirt body [52]. The purity of all filtrated hydrolysate was escalated ranging from 1.14-2800 folds when compared to the native hydrolysate. For example, the purity of hydrolysate of Pangasius catfish skin and bone were increased around 550 and 1200 folds after fractionation via membrane MWCO of 1000 Da, respectively [51]. After UF with membrane MWCO < 2500 Da, the hydrolyste from Loach meat showed the IC_{50} = 0.231 mg/ml decreased from native hydrolysate (IC_{50} = 0.631 mg/ml) resulting in the purity increased around 3000 times [11]. Hence, it can be concluded that UF improved the activity of ACEIP by taking out some of hydrolysate that have high molecular mass and no ACE inhibitory activity [5].

Gel filtration (GF)

In GF, ACEIPs are fractionated based on their relative size [23]. One of the most widely used column is Sephadex for example, Sephadex G-15 for MW<1500 Da and G-25 for MW < 1000-5000 Da [11-13, 40, 43]. Beside, GF is also used to determine the MW of ACEIP or remove low MW impurities. From Table 1, the lower MW of hydrolysate obtained from GF than UF due to the more specificity of column used. MW of hydrolysates from aquatic resources ranged 380-1320 Da [27, 34, 40, 42, 43]. Besides, the much lower IC_{50} value of hydrolysate after this step compared to native hydrolysate derived was shown. For instance, the study of Zhao et al. [8] showed that the purity of hydrolysate of sea cucumber was improved 6.58-fold with recovery of 28% after using SephadexG-15 gel filtration.

Besides, after Sephadex G-25 gel filtration chromatrography, the IC_{50} of hydrolysate of fresh water clam solid wastes (MW, 380 Da) was decreased from 650 µM to 59 µM representing that 11-fold of purification [43]. Thus, GF is another purification method that specific MW and higher ACE inhibitory activity were obtained.

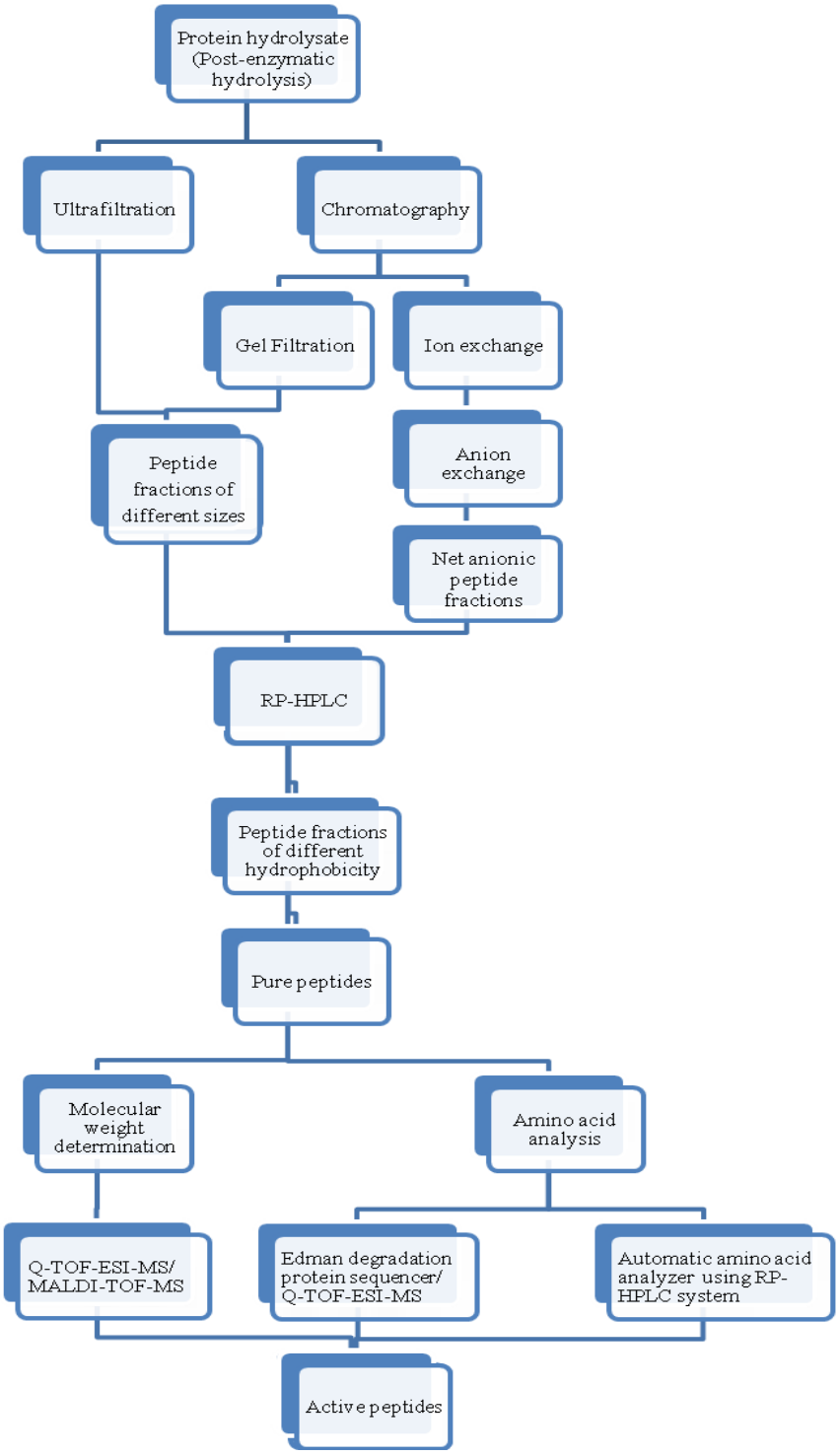

Figure 2. Schematic diagram showing steps toward the purification and identification of aquatic resources derived bioactive peptides

Table 1. Purification methods used for ACE inhibitory peptides from aquatic resources

Method	Peptides	MW (Da)	IC_{50} [a]	IC_{50} [b]	Yield (%)	Purity (fold)	Origin/Enzyme	Reference
Ultrafiltration	Gly-Pro-Ala-Met	<1000 <1000	1680 1550 µg/ml	3.2 1.3 µg/ml	-	525 1,192	Pangasius catfish skin/Alcalase Pangasius catfish bone /Alcalase	[51]
	Ala-His-Leu-Leu	<2500	653.3 µg/ml	0.231 µg/ml	78	2,828	Loach muscle/Bromelain	[11]
	1.Gly-Pro-Pro-Pro-Pro 2. Ile-Glu-Lys-Pro	<3000 <3000	8.02 10.54 mg/ml	1.36 2.10 mg/ml	-	5.89 5.02	-Catfish sarcoplasmic protein -Catfish myofibrillar protein /Thermolysin	[46]
	Met-Leu-Leu-Cys-Ser	<5000	1.17 mg/ml	0.828 µM	29.7	1.41	Sea squirt body/ Protamex	[52]
Gel filtration chromatography	Met-Glu-Gly-Ala-Gln-Glu-Ala-Gln-Gly-Asp	840	0.615 mg/ml	0.0934 mg/ml	28.4	6.58	Sea cucumber flesh/Bromelain -Alcalase	[7, 8]
	Leu-His-Pro	311-1320	1.17 mg/ml	0.77 mg/ml	10.3	1.52	Marine shrimp meat/Pepsin	[40]
	Hyp-Pro	-	1.19 mg/ml	0.39 mg/ml	13.09	3.05	Cuttlefish skin gelatin/Cuttle fish hepatopancreas protease	[18]
	Val-Ile-Tyr	-	0.57 mg/ml	0.096 mg/ml	-	5.94	Sea bream skin/Protease from *Bacillus subtilis*	[38]
	Met-Leu-Leu-Cys-Ser	566.4	1.771 mg/ml	0.39 mg/ml	-	3	Ascidian body/Protamax	[52]
	1. Val-Trp-Asp-Pro-Pro-Lys-Phe-Asp 2. Phe-Glu-Asp-Tyr-Val-Pro-Leu-Ser-Cys-Phe 3. Phe-Asn-Val-Pro-Leu-Tyr-Glu	602.30 469.30 147.05	365 µg/ml	139 µg/ml	-	2.63	Salmon pectoral fin/Alcalase	[16]
	1. Pro-Gly-Pro-Leu-Gly-Leu-Thr-Gly-Pro 2. Gln-Leu-Gly-Phe-Leu-Gly-Pro-Arg	975.38 874.45	0.68 mg/ml	0.21 mg/ml	-	3.24	Skate skin/ α-chymotrysin	[41]

Method	Peptides	MW (Da)	IC_{50} [a]	IC_{50} [b]	Yield (%)	Purity (fold)	Origin/Enzyme	Reference
Gel filtration chromatography	1. Val-Lys-Pro 2.Val-Lys-Lys	380-420	650.72 µM	58.51 µM	-	11.12	Fresh water clam solid wastes /Crude protease from *Aspergillus oryzae* and *Rhizopus oryzae*	[43]
Ion exchange chromatography	Leu-Leu-Met-Leu-Asp-Asn-Asp-Leu-Pro-Pro	1,301	60.40%[c]	71.81%	28.8	1.19	Pacific cod skin/Gastrointestinal protease	[34]
	1.Thr-Cys-Ser-Pro 2.Thr-Gly-Gly-Gly-Asn-Val	485.5	-	81%	-	-	Pacific cod skin/Papain	[27]
	1. Val-Trp-Asp-Pro-Pro-Lys-Phe-Asp 2. Phe-Glu-Asp-Tyr-Val-Pro-Leu-Ser-Cys-Phe 3. Phe-Asn-Val-Pro-Leu-Tyr-Glu	602.30 469.30 147.05	365 µg/ml	169 µg/ml	-	2.16	Salmon pectoral fin/Alcalase	[16]
	1.Thr-Phe-Pro-His-Gly-Pro 2.His-Trp-Thr-Thr-Gln-Arg	744 917	86.2%	0.068 1.44 mg/ml	-	-	Seaweed pipefish muscle/Alcalase	[54]
RP-HPLC	1. Ala-Pro 2. Val-Arg	186.18 273.30	1.17 mg/ml	0.06 0.332 mg/ml	-	19.5 3.5	Atlantic salmon skin/Alcalase-papain	[19]
	Val-Glu-Gly-Tyr	467.2	1.47 mg/ml	0.06 mg/ml	0.08	24.5	Marine seaweed protein/Alcalase	[54]
	Asp-Asp-Thr-Gly-His-Asp-Phe-Glu-Asp-Thr-Gly-Glu-Ala-Met	1538	0.63 mg/ml	14.82 µg/ml	-	42.5	Rotifer body/Alcalase	[22]
	1. Tyr-Ala-Pro, 2. Val-Ile-Ile-Phe 3. Met-Ala-Trp	407.2 448.2 491.2	1.19 mg/ml	0.006 0.0027 0.0042 mg/ml	-	180.30 440.74 283.33	Cuttlefish skin gelatin/Cuttlefish hepatopancreas protease	[18]
	Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	1195	9.5 µmol/ml	0.066 µmol/ml	8.5	144	Oyster mantle/Pepsin	[12]
	Phe- Arg- Gly- Leu-Met-His-Tyr	200 - 600	1.2 mg/ml	0.81 mg/ml.	-	1.48	Sardinelle heads /Alkaline protease	[49]

Method	Peptides	MW (Da)	IC50a	IC50b	Yield (%)	Purity (fold)	Origin/Enzyme	Reference
RP-HPLC	1.Phe-Thr-Tyr 2.Phe-Ser-Tyr	-	0.075 0.035 mg/ml	0.0009 0.0255 mg/ml	-	83 1.37	Shrimp by-product/Alcalase	[21]
	1. Gly-Pro-Pro-Pro-Pro 2. Ile-Glu-Lys-Pro	<3000 <3000	8.02 10.54 mg/ml	67% 62%	-	-	-Catfish sarcoplasmic protein -Catfish myofibrillar protein hydrolysate/Thermolysin	[46]
Two step RP-HPLC	Val-Ala-Pro	<3000	0.872 mg/ml	1st 0.0553 2nd 0.00534 mg/ml	-	15.77 163.30	Grass carp muscle/Alcalase	[17]
	Met-Ile-Phe-Pro-Gly-Ala-Gly-Gly-Pro-Glu-Leu	130	0.883 mg/ml	1st 0.056 2nd 0.029 mg/ml	-	15.8 30.4	Yellowfin sole frame protein/ α-chymotrypsin	[15]
	Met-Leu-Leu-Cys-Ser	566.4	1.771 mg/ml	1st 0.056 2nd 0.014 mg/ml	-	20.91 83.64	Ascidian body/Protamax	[52]

IC_{50} = concentration of sample that inhibit 50 % of ACE activity
[a] IC_{50} value of ACE inhibitory peptides before purification
[b] IC_{50} value of ACE inhibitory peptides after purification
[c] Reported as ACE inhibitory Activity (%)

Table 2. Purification and identification methods for ACE inhibitory peptides from aquatic resources

Purification		Reference
Method	**Characteristic**	
Molecular weight		
Ultrafiltration	Membrane with specific MWCO (1 kDa, 2.5 kDa, 3 kDa,5 kDa)	[6-10]
Gel filtration chromatography	Sephadex G-15 (1.6cm×68cm), (1.6 cm×100 cm)	[6, 8, 11-13,16, 42, 54]
	Sephadex G-25 (1.6 cm × 90cm), (5 cm × 57 cm)	[11, 19, 39]
	Sephadex LH-20 (2.7 cm×80 cm)	[6-8, 11, 16, 40, 42, 54]
	DA 201-C macroporous resin	[6, 10, 18, 39, 52]
Consecutive chromatography		
Ion exchange chromatography	SP-Sephadex C-25 (35 mm × 350 mm)	[15]
	Hypersil BDS C18 (4.6 mm × 210 mm,5 μm)	[12]
	HiPrep 16/10 DEAE FF	[16, 34, 54]

RP-HPLC	Vydac C18 column (10 mm × 250 mm)	[18]
	XBridge BEH130 C18 column (4.6×250 mm)	[19]
	SunFire C18 column (4.6 × 250 mm)	[40]
	YMC-Pack ODS-A column (C18, 5 µm, 4.6 × 250 mm)	[52]
Two step RP-HPLC	1st: SP Nucleosil 100-7 C18 (1.0 × 250 mm) 2nd a Zorbax SBC18 (4.6 × 250 mm)	[15]
	1st: a Primesphere 10 C18 (20 ×250 mm) 2nd: a YMC-Pack Pro C18 (20×250 mm)	[34]
	1st a Jupiter C18 column (5 µm, 10 mm×250 mm) 2nd: a Luna C18 column (5 µm, 4.6 mm×250 mm)	[17]
Identification		
Characteristic	**Method**	**Reference**
Molecular weight	SDS-PAGE	[9,39]
	Ultrafiltration by membrane with specific MWCO (1 kDa, 2.5 kDa, 3 kDa,5 kDa)	[6-10]
	Q-TOF mass spectrometer coupled with an electrospray ionization (ESI) source	[6, 8, 11-13,16, 42, 54]
Amino acid composition	RP-HPLC by a C18-reversed phase column (Thermal C18 5U, 250 × 4.6mm)	[11, 19, 39]
Amino acid sequence	Q-TOF mass spectrometer coupled with an electrospray ionization (ESI) source	[6-8, 11, 16, 40, 42, 54]
	Electrospray ionisation-mass spectrometry (ESI-MS) and the tandem mass spectrometry (ESI-MS/MS)	[6, 10, 18, 39, 52]

Ion exchange chromatography (IE)

IE is widely used at the beginning of a purification scheme and is designed for the separation of ionic or ionizable compound in the mobile phase by the counter-ion of the opposite sign in the stationary phase (column packing) [23]. Among the fixed stationary phase charge, diethylaminoethyl (DEAE) is widely used in conventional anion exchange chromatography during ACEIP purification [14, 16, 20, 54]. Wijesekara et al. [14] used IE to purify hydrolysate from seaweed pipefish muscle and obtained the reasonable IC_{50} value. Ahn et al. [16] used a Hiprep 16/10 DEAE ion exchange column to purify the hydrolysate of salmon pectoral fin. They found that the purified hydrolysate provides IC_{50} = 169 µg/ml after getting through the column and decreased around three times when compared to the native hydrolyaste (IC_{50} = 365 µg/ml). IE seem to be narrowly used in ACE inhibitory peptide purification due to the structure-relativity with ACE depending on hydrophobicity and hyprophilicity.

Reversed phase high performance liquid chromatography (RP-HPLC)

RP-HPLC has been widely used for separation and analysis for ACEIP [18, 19, 21, 46, 49, 52, 54]. Like standard RP chromatography, bonded phases like C-18 was available for separation of ACEIP by RP-HPLC. Typically, columns with particle sizes of 2-10 µM are used in RP-HPLC. Samples are eluted at constant flow rates with a linear gradient of solvent in column, and amino acid fractions are recollected automatically. Major peaks are numbered and the following sequences can be identified in the chromatogram [3, 55]. The second RP-HPLC was done to obtain exceedingly pure ACEIP using the same or different column [11, 16, 20]. The most purity of peptide derived from this step because of the efficacy of specific column used. Ko et al. [54] isolated and purified ACEIP from marine seaweed. They found the peptide sequence of Val-Glu-Gly-Tyr with MW of 467.2 and IC_{50} of 0.06 mg/ml after using ODS C-18 RP-HPLC. Besides, recovery and purity of the peptides was calculated as 0.08% and 24.5-fold, respectively. After RP-HPLC, the purified peptides derived from cuttlefish skin gelatine hydrolyzed by cuttlefish hepatopancreas protease showed 440-fold when compared to native one [18]. The pepcidic peptide of oyster mantle with MW of 1195 Da showed the purity up to 144 fold when compared to the native one [12]. In case of pharmalogical purpose, very high purity of ACEIP is required. So, some researchers used two steps chromatography to obtain high purity of ACEIP [15, 17, 52]. In Grass carp muscle hydrolyzed by Alcalase, the purified peptides exhibited IC_{50} of 0.0553 and 0.00534 mg/ml in first and second RP-HLPC, respectively resulting in the purity increased more than 10 times [17]. The same trend of increasing purity was also seen from the study of Ko et al. [52] found that the purified peptide showed 4 times increasing compare to the first purification.

Identification of ACE inhibitory peptides

After obtaining purified ACEIP from the purification step, determining for MW and amino acid composition or amino acid sequence need to be performed. The identification of the structure of peptides is used to describe their amino acid sequence. Recent studies on bioactive peptides are aimed to clarify the structure-activity relationship of peptides, which is essential information for the design of novel therapeutics or functional food ingredients [5]. Individual ACEIP fractions are identified using the combined techniques of mass spectrometry, HPLC, and Edman degradation protein sequencing. Amino acid composition or sequence, MW as well as IC_{50} value of aquatic resources derived ACEIP were summarized and presented in Table 1. Furthermore, identification method used for ACEIP production from aquatic resources was summarized in Table 2.

Determination of molecular weight (MW)

Researchers have identified specific peptides responsible for ACE activity. The size of the generated peptides is crucial on the ACE inhibitory effect as previously reported [56, 57]. The peptides with lower MW are easily absorbed compared to peptides with higher MW [58]. Hence, identification for MW is very important. Qualitative methods namely sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) can be used for studying MW of ACEIP [8, 9]. The SDS-PAGE results showed that Alcalase hydrolysate of sea cucumber represented a unique pattern compared to others, which yielded potent MW distribution lower than 20 kDa [8]. Apart from SDS-PAGE, the MW distribution of the hydrolysates was determined by ultrafiltration techniques with difference MWCO and/or size exclusion chromatography [6, 9, 20].

For quantitative methods, an accurate molecular mass of purified ACEIP was determined by using mass spectrometry methods. An quadrupole time-of-flight mass spectroscopy coupled with electrospray ionization (ESI) source (Q-TOF-ESI-MS) [6, 8, 11, 16, 42, 54] and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS) [9, 39] are two main techniques for measuring molecular mass.

For the former, the purified ACEIP in solution form is pumped through a narrow, stainless steel capillary; a high voltage is applied to the tip of the capillary, which is located within the ionization source of the mass spectrometer, and a stream of nebulising gas is introduced. As a consequence of the strong electric field, the sample emerging from the tip is sprayed into highly charged droplets.

These droplets are then evaporated at the interface by the drying gas. Charged sample ions, free from solvent, are finally released from the droplets and detected by the analyzer. For the latter, the dried purified ACEIP is introduced into the mass spectrometer where a laser is fired to desorb and ionize the sample. The matrix is used to absorb the energy needed for samples desorption and ionization from the laser and the analyzer separates ions according to their mass-to-charge ratio. A tandem mass spectrometer (MS/MS) has more than one analyzer and generates structural information for a compound by fragmenting specific peptide ions and identifying the resulting fragment ions [59]. For example, MW distribution of the hydrolysates was determined by size exclusion chromatography and MALDI-TOF-MS, which analyzed a representative hydrolysate from pacific cod skin gelatin with a weight range of 1000-3000 Da. The MW of 1301 Da was determined [34]. The MW of 566.4 Da and amino acid sequence of Met-Leu-Leu-Cys-Ser the purified peptide from *S. plicata* was determined using a Q-TOF mass spectrometer [52].

Determination of amino acid composition

Since inhibitory effect of a peptide is affected by the type of amino acids present in its N- or C-terminal sequence which totally depend on the original primary structure and the choice of enzyme [39, 56, 57]. Forgani et al [39] suggested that position of those amino acids in the peptide sequence is more crucial than their total amounts. Amino acid composition was studied using RP-HPLC system in qualitative method [39]. For quantitative method, automatic amino acid analyzer was used [11, 19]. After studying amino acid composition by using RP-HPLC system, Forghani et al. [39] found that the amino acid profile of untreated sea cucumber showed that Gly dominated among others with content of 66.2 mg/g. Glu was the second most abundant component (53.8 mg/g) followed by Ala and Pro with contribution of 52.5 and 48.2 mg/g, respectively. For example, Atlantic salmon skin peptides containing with in Gly (15.94%), Pro (8.68%), Ser (5.52%), Ala (8.04%), Glu (13.90%) and Asp (9.45%) were analyzed from automatic amino acid analyzer [19]. The composition derived from both sea cucumber and salmon fin peptides showed high in Pro, Ala and Gly that compatible with the properties of ACE inhibitor. As mentioned before, it is known that hydrophobic, aromatic or branched side chains, (Pro) and positively charged amino acids (Lys or Arg) existing in C-terminal contributes to the ACE inhibitory properties [39].

Determination of amino acid sequence

Amino acid sequence can be defined by either automated Edman degradation protein sequencer [13, 15] or Q-TOF-ESI-MS [6-8, 11, 16, 40, 42, 54]. ESI-MS and MS/MS were also used for detecting amino acid sequence of purified ACEIP [6, 10, 39, 52]. For example, the purified peptide of Pacific cod skin gelatin hydrolysate was analysed by Q-TOF-ESI mass spectroscopy to find the amino acid sequence and it was identified as Leu-Leu-Met-Leu-Asp-Asn-Asp-Leu-Pro-Pro with IC_{50} 35.7 µM [36]. Ko et al. [52] studied amino acid sequence of ACEIP from sea squirt, *Styela plicata*. Amino acid sequences were identified using MS/MS and proved to be a penta peptide, Met-Leu-Leu-Cys-Ser with ACE inhibitory IC_{50} values of 24.7 µM, and MW 566.4 Da. The peptide (Met-Ile-Phe-Pro-Gly-Ala-Gly-Gly-Pro-Glu-Leu) from yellowfin sole frame showed IC_{50} value of 22.1 µM [15], *Acaudina molpadiodea* (Met-Glu-Gly-Ala-Gln-Glu-Ala-Gln-Gly-Asp) having IC_{50} value of 15.9 µM [8].

Physiological function of bioactive peptides

Peptides generated by the digestion of various proteins, including animal, plant and microbial sources, possess biofunctional activity [26, 35]. These peptides are inactive while part of the sequences of their parent proteins, but are released during enzymatic hydrolysis, gastrointestinal digestion or fermentation and/or food processing [60, 61].

Once such bioactive peptides are liberated, depending on their structure, composition and sequence, they may exhibit various biofunctional activities [26, 36]. Bioactive peptides have been discovered with various biofunctional activities for instance, antihypertensive [24-26, 30, 31, 34, 38-43, 46, 47, 49], antioxidative [10, 28, 31], antimicrobial [29], cholesterol-lowering [30, 31], anticancer [32, 33], cellular oxidation stress inhibition [34], antithrombotic, mineral absorption enhancement, and immunomodulatory properties [35, 36]. Biofunctional activities of bioactive peptide derived from aquatic resources were summarized in Table 3.

Table 3. Biofunctional activities of bioactive peptides from aquatic resources

Functional	Source	Reference
Antihypertensive	Cod skin, salmon skin, clam mantle, catfish muscle, catfish skin and bone	[30, 31, 34, 38-43, 46, 47, 49]
Antioxidative	Brownstripe red snapper muscle	[28]
	Squid inner and outer tunics	[10]
	Blacktip shark skin	[31]
Hypocholesterolemic	Blacktip shark skin	[31]
Antimicrobial	Leatherjacket insoluble muscle	[29]
Anticancer/ Antiproliferative	Tuna dark muscle Pacific cod skin	[32, 33]
Cellular oxidation stress	Pacific cod skin	[33]
Inhibition DNA oxidation	Blacktip shark skin	[31]

CONCLUSION

Peptides that generated by the enzymatic hydrolysis of various aquatic resources was determined for ACE inhibitory activity and then subjected to purify, identify, to yield extra purity and efficacy ACEIP. The derived ACEIP after purification and identification are very active and offer strong various biofunctional activity.

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