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RESEARCH PAPER

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In Vitro Stability of ACE-Inhibitory Peptides of Rohu Fish Waste to Heat, pH and Gastrointestinal Enzymes

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Abstract

Angiotensin-I converting enzyme inhibitory (ACE*i*) peptides were prepared from rohu (*Labeo rohita*) fish wastes by enzymatic hydrolysis [1.1% alcalase concentration (v/w); 130 min time; 52°C temperature and 0.8:1 solid-liquid ratio]. Ultrasound Assisted Enzymatic Extraction (UAEE) and Microwave Assisted Enzymatic Extraction (MAEE) were performed to enhance degree of hydrolysis (DH), ACE*i* activity, and peptide yield (PY). The ACE*i* peptides were examined for stability during thermal processing, varied pH treatments and *in vitro* gastrointestinal digestion. Peptides were stable at different temperatures (25, 37, 55, 75, and 100°C) except at 121°C which reduced their stability by 7%, 6.9% and 2.2% in UAEE, MAEE and Non-Assisted Enzymatic Extraction (NAEE) peptides, respectively. Similarly, peptides retained ACE*i* at pH 2 to 8 and subsequent reduction at pH>8. *In vitro* digestion studies showed stability of all peptides to GI digestion at 0.05% pepsin, 0.05% trypsin, 0.05% chymotrypsin and the combination of 0.025% trypsin and 0.025% chymotrypsin except MAEE peptides showing less stability with pepsin (0.05%). Therefore, it is concluded that these ACE*i* peptides derived from rohu fish waste, stable to heat, pH and gastrointestinal enzymatic action, can be used as ingredient in functional foods, which undergo thermal processing and digestion.

Introduction

Bioactive peptides are specific sequences of protein having 2-30 amino acids that impart various health benefits to the consumers upon ingestion (Lafarga and Hayes, 2014). Various bioactivities of peptides include anti-hypertensive, opoid, anti-oxidant, anti-thrombic, anti-adipogenic, antimicrobial and anti-inflammatory properties (Lafarga and Hayes, 2014; Decastro and Sato, 2015). Bioactive peptides are found in a wide range of animal foods like milk, beef, chicken, fish, pork, and plant foods. Recently, food derived bioactive peptides attract great attention because of their multiple health benefits and advantages over traditional drugs (Nasri *et al*., 2013).

In the food industry, functional food with anti-hypertensive peptides is a fast-growing segment with larger expansion day by day due to the increase in awareness and purchasing power of the consumers (Zuhaib *et al*., 2017). Anti-hypertensive or ACE-

inhibitory (ACE*i*) peptides find special attention because of their positive response in the treatment of hypertension, which otherwise leads to stroke, myocardial infarction, cardiac failure, and dementia. ACE-inhibitory peptides are used in foods as a nutraceutical or functional food for health promotion (Liu *et al*., 2018). Collagen peptides attracted much attention recently due to their high biocompatibility, low toxicity, and high bioactivity (Sun *et al*., 2022).

In vitro and *in vivo* studies are used to demonstrate the effect of various dietary proteins and peptides in improving human health and biological function, including anti-hypertensive activity (Nongonierma and Fitzgerald, 2017). The challenge before food technologists, researchers, clinicians, and pharmacists is to ensure the delivery and bioavailability of peptides. Bioactive peptides get hydrolyzed at different stages of gastrointestinal digestion and then get absorbed by the intestinal epithelium (Picariello *et al*., 2010). The Gastrointestinal (GI) tract is one of the known barriers in the human body that decides the actual digestion, absorption, and assimilation of nutrients. The conditions in the gastrointestinal tract, such as digestive enzymes and pH affect the structure and function of peptides (Toldra *et al*., 2020).

Bioactive peptides need to be stable when subjected to food processing when added as a functional ingredient in the food (Ramakrishnan *et al*., 2023). Before ingestion, bioactive peptides incorporated in functional food are subjected to various processing involving heat treatments. Upon ingestion, bioactive peptides will have to undergo hydrolysis by digestive enzymes and by extreme pH in the GI tract. It is therefore important to evaluate the stability of bioactive peptides to GI barriers such as processing temperature, pH, and digestive enzymes in retaining the ACE*i* activity. The thermal stability of bioactive peptides ensures their stability when they undergo several heat treatments at the time of processing. Stability of peptides against pH helps to know their activity during passage through the GI tract in acidic and alkaline pH at the time of digestion. The stability of peptides against GI digestive enzymes is crucial to know about additional hydrolysis and the bioactivity of the resultant peptides (Seladi-Schulman, 2022).

Previous studies have reported that *in vitro* digestion assay is a useful tool to analyze the bioavailability and digestibility of bioactive compounds, including peptides (Bougatef *et al*., 2008; Balti *et al*., 2010; Nasri *et al*., 2013; Toopcham *et al*., 2015). Bougatef *et al*. (2008) fractionated the protein hydrolysate (IC50=1.2±0.09 mg/ml) generated from enzymatic hydrolysis of sardine viscera using alkaline proteases by size exclusion chromatography and obtained eight major fractions (P1–P8). Out of these, fraction P4 exhibited ACE inhibitory activity (IC50=0.810.013) and showed *in vitro* digestion by GI proteases. GC/MS analysis revealed that P4 was rich in phenylalanine, arginine, glycine, leucine, methionine, histidine, and tyrosine. Balti *et al*. (2010) did not find an apparent change in ACE-inhibitory activities of protein hydrolysates prepared from cuttlefish (*Sepia officinalis*) muscle after *in vitro* incubation with gastrointestinal proteases. There was no change in the ACE-inhibitory activity of cuttlefish (*Sepia officinalis*) muscle peptides before and after a temperature treatment of 4 to 100°C for 2 h

(Balti *et al*., 2010). Heat treatment (90°C, 240 min) did not change the bioactivity of Goby (*Zosterissessor ophiocephalus*) fish protein hydrolysates (Nasri *et al*., 2013). A fraction (P1) obtained from tilapia mince (TM) hydrolyzed by *Virgibacillus halodenitrificans* SK1-3-7 proteinases retained its ACE inhibitory activity after severe thermal treatments (100°C or 121°C). Additionally, the same study found inhibitory activity to remain constant at pH 2-10. ACE inhibitory peptides from tilapia also exhibited resistance to *in vitro* gastrointestinal digestion suggesting it for a potential therapeutic use because of its resistance to gastrointestinal digestion (Toopcham *et al*., 2015).

Ultrasound-assisted enzymatic extraction (UAEE) and microwave-assisted enzymatic extraction (MAEE) are recently applied to produce bioactive peptides with an enhanced degree of hydrolysis (DH), ACE inhibition (ACE*i*), and peptide yield (PY) by several authors (Jia *et al*., 2010; Huang *et al*., 2014; Nguyen, 2016; Hall and Liceaga, 2020). In this study, peptides derived from rohu fish wastes using alcalase following UAEE, MAEE and non-assisted enzymatic extraction (NAEE) were assessed for their *in vitro* stability to various temperatures, pH and digestive enzymes in retention of ACE*i* activity.

Materials and Methods Extraction of ACE inhibitory Peptides

Eighteen kilograms of rohu (*Labeo rohita*) fish were procured from the local fish market near Gowsala, Hambran road, Ludhiana, Punjab (India). Fish waste was separated by firstly descaling using a descaling hand tool followed by dressing using a fish cleaning-cum-waste collection system. The fish waste (49.4% of total fish weight) obtained from 3 lots of fish, each with six kilograms rohu were subjected to treatment. One lot was used to extract peptides using alcalase, following the previously standardized condition at an enzyme concentration of 1.1% (v/w); a temperature of 52°C; hydrolysis time of 130 min and a solid-liquid ratio of 0.8:1 (Kumar *et al*., 2022). Peptides obtained without assistance were termed non-assisted enzymatic extraction (NAEE) peptides. Another lot was pre-treated with ultrasound using a bench top bath type sonicator (CUB10, M/s ACZET, Mumbai, India) at 40 kHz frequency, 63 min sonication time and 58°C sonication temperature and subjected to enzymatic extraction. The resultant peptides are termed UAEE peptides. The third lot was pretreated with microwave at a frequency of 2450 MHz at the standardized power of 335 W for 15

min in a microwave oven (MC3286BRUM, LG Electronics, India) and then subjected to enzymatic hydrolysis. The derived peptides were termed MAEE peptides. Peptides were then sequentially filtered through syringe filters of 0.45 µm and 0.20 µm (M/sHimedia laboratories, Mumbai, India) followed by molecular weight cut-off ultrafilters (MWCO, 10, 5, and 3 kDa; M/s Sartorius Stedium Lab, UK and/s Merck Millipore, Germany) to obtain low MW peptides. Peptides were then lyophilized in a lyophilizer (MAC MSW- 137, FD-6, New Delhi, India), kept in an airtight vial and wrapped in an aluminium foil to prevent desiccation on storage until used for analysis.

Degree of Hydrolysis

Degree of hydrolysis was determined by the method of Adler-Nissen (1979). Fish peptide was added with PBS (0.2125 M, pH 8.2) and TNBS (0.1%) and incubated in the dark at 50°C for 1 h. The reaction was terminated using HCl (0.1 M) and the absorbance was measured at 340 nm in a spectrophotometer and expressed as α-amino acids in terms of L-Leucine.

$$DH\ (\%) = ((Lt\text{-}Lo)\times 100)/(Lmax\text{-}Lo)$$

where, Lt: amount of α-amino acids released at time 't'; Lo: amount of α-amino acids released in acid-solubilized substrate and Lmax: amount of maximum α-amino acids found

ACE-inhibition Assay

ACE*i* activity was assessed as per the modified method of Murray *et al*. (2004) and Theodore and Kristinsson (2007). N [3-(2-Furyl)acryloyl]-Phe-Gly-Gly (FAPGG) was used as the synthetic substrate for the assay. Peptides (100 µL, 0.2% protein w/v), enzyme (50 µL, 15 mU ACE) and substrate (1 mL, 0.5 mM FAPGG) were incubated at 25°C in a water bath for 60 min.

The absorbance was measured at 334 nm using a UV-Vis Spectrophotometer (UV3200, Labindia, Mumbai). FAPGG with ACE served as a control. Hydrolysis of FAPGG by ACE results in a decrease in absorbance value. The ACE*i* activity of peptides was measured by their ability to decrease the hydrolysis of FAPGG. A complete inhibition of ACE indicates a 100% ACE inhibition.

$$ACE\ inhibition\ (ACEi)\ (\%) = \left(1 - \frac{Slope\ of\ sample\ curve}{Slope\ of\ control\ curve}\right) \times 100$$

Peptide Yield

Peptide yield was determined as per the method of Wu *et al*. (2019). ACE-inhibitory peptides were dissolved in trichloroacetic acid (10%), kept at room temperature for 20 min and then centrifuged at 10000g, 10 min. The supernatant was collected to determine peptide content by the biuret method using Bovine Serum Albumin (BSA) as standard. Peptide yield was calculated in percentage considering conversion coefficient of nitrogen to protein as 6.25.

$$\text{Peptide Yield (PY) (\%)} = \frac{(\text{TCA}-\text{Soluble Peptide Content})\times 100}{\text{Total Protein Nitrogen}\times 6.25}$$

***In Vitro* Stability Studies**

The heat stability of NAEE, UAEE and MAEE peptides was studied as per the method of Toopcham *et al*. (2015). Peptides (0.75 mg/ml) prepared in distilled water were held at 25, 37, 55, 75 and 100°C for 2 h and at 121°C for 15 min. A water bath (M/s MAC Macro Scientific Works, New Delhi, India) was used to maintain temperature for 25-100°C and an autoclave (Model: SVA Scientific Automatic Autoclave, Scientific Systems, New Delhi) was used to achieve 121°C. Peptides incubated at different temperatures were then cooled to room temperature (25°C) and examined for their ACE*i* activity.

The pH stability of peptides was analyzed by incubating extracted peptides (0.75 mg/mL, w/v) at five different pH conditions *viz.* 2, 4, 6, 8, and 10, and holding them at 40°C for 2 h at their respective pH. After the incubation, the pH of the samples was adjusted to 8.3 and then tested for ACE*i* activity. Stability of peptides to digestive enzymes *viz.* pepsin, trypsin, and chymotrypsin (Sigma Aldrich, USA) was examined following the *in vitro* digestion method of Bougatef *et al*. (2008). Peptides (10mg/mL) prepared in deionized water were individually digested using 0.05% (w/v) pepsin (pH 2.0), trypsin (pH 8.0) and chymotrypsin (pH 8.0) for 3 h at 37ºC. Another successive enzymatic digestion was performed by incubating peptides first in trypsin (0.025% w/v) for 3h and then digesting them with chymotrypsin (0.025% w/v) for 3h at 37ºC. The enzyme reaction was terminated at each step by heating the digestive mixture in boiling water for 5 min. The resultant digested mixture was centrifuged at 10,000×g for 10 min in a refrigerated centrifuge (Z326K; HERMLE, Labor Technik, Wehingen, Germany) and the supernatant was used to examine for ACE*i* activity.

Statistical Analysis

All experiments were done in triplicate and expressed as the mean ± standard deviation (n=3). Differences between variables (extraction methods, heat, pH and gastrointestinal enzyme treatments) in respect of ACE*i* activity were tested for significance by one-way analysis of variance using Microsoft Excel 2019 (version 2112). When the value of $P<0.05$, the differencewas considered significant. The source of variation considered were between treatments of each of heat, pH and GI enzyme and also between extraction methods (NAEE, UAEE and NAEE).

Results and Discussion

Degree of Hydrolysis, ACE inhibition and Peptide Yield of Peptides

Table 1 shows degree of hydrolysis (DH), ACE inhibition (ACE*i*) and peptide yield (PY) of peptides obtained by NAEE, UAEE and MAEE. The highest DH and PY were obtained for MAEE peptides while the highest ACE*i* was found for UAEE peptides. There was significant (P<0.05) difference in the extraction efficiencies of both UAEE and MAEE methods over NAEE method. However, there was no significant difference (P>0.05) among UAEE and MAEE in respect to DH, ACEi and PY. With respect to ACE*i*, UAEE peptides showed better performance over MAEE peptides. Conversely, MAEE peptides showed better efficiencies in respect of DH and PY.

In vitro stability studies

In vitro stability of NAEE, UAEE and MAEE peptides evaluated during hydrolysis at different heat treatment (25 to 100°C for 2 h and 121°C for 15 min), pH (2 to 10) and gastrointestinal enzymes (pepsin-0.05%, trypsin-0.05%, chymotrypsin-0.05%, and trypsin-0.025% + chymotrypsin-0.025%) showed that all peptides had good ACE*i* between 25°C to 100°C, maximum ACE*i* between pH 6-8 and minimum at pH 10. Additionally, MAEE peptides showed low ACE*i* at pH 2-4. All peptides retained ACE*i* against all GI enzymes, except MAEE peptides that showed low activity for pepsin. The study inferred that both UAEE and MAEE can produce peptides with enhanced ACE*i* of peptides, however, UAEE is recommended to derive more stable ACE-inhibitory peptides from rohu fish wastes. Our results indicated that the ACE*i* activity of NAEE, UAEE and MAEE peptides was 55±0.70%, 60±0.60%, and 59±0.80% respectively before undertaking the stability studies. Analysis of variance was calculated for *in vitro* stability of peptides against heat treatments, pH treatments and GI enzymes treatments. The source of variation for statistical analysis were between heat treatments, pH treatments and GI enzyme treatments as well as between extraction methods (NAEE, UAEE, MAEE). UAEE (Figure 1A, 2A, 3A) and MAEE (Figure 1B, 2B, 3B) peptides possessed higher ACE*i* activity (P<0.05) as compared to NAEE (Figure 1C, 2C, 3C) peptides. However, the ACE inhibition between UAEE and MAEE peptides was insignificant (P>0.05).

Stability to Heat

The study on the thermal stability of bioactive peptides is important to ensure their activity when they undergo heat treatments during processing. High temperature is known to alter peptide structures resulting in the degradation of peptides into fragments and thereby lose their bioactivity (Wang *et al*., 2017). In this study, peptides subjected to different heat treatments showed that peptides were thermal stable up to 100°C, while at 121°C, the ACE*i* activity decreased. The NAEE peptides (Figure 1C) retained ACE*i* between 25°C (55.0±0.5%) and 100°C (54.7±1.0%). However, ACE*i* significantly (P<0.05) reduced beyond 121°C (53.8±1.3%). Similarly, the UAEE peptides (Figure 1A) retained the ACE*i* between 25°C (59.2±0.9%) and 100°C (59.1±0.5%) without any significant change in ACE*i* values (P>0.05). However, there was a significant reduction (P<0.05) in ACE*i* activity of UAEE peptides at 121°C (55.8±0.6%). Thus, the UAEE peptides exhibited good thermal stability up to 100°C but not above 121°C. The sterilization temperature of 121°C destroyed peptides within a shorter time duration of 15 min. The MAEE peptides (Figure 1B) also exhibited a similar trend but with slightly low thermal stability (P>0.05) as compared to the UAEE peptides (Figure 1A). The MAEE peptides showed a significantly (P<0.05 marginal reduction in ACE*i* (P>0.05) from 25°C (59.3±0.7%) to 100°C (58.5±0.4%), while the reduction was significant at 121°C (54.9±1.4%) (P<0.05). At the higher temperature of 121°C, the reduction in ACE*i* was more pronounced in UAEE peptides (4.2%) (ACE*i* of controlled UAEE peptide was 60%) (Figure 1A) followed by MAEE peptides (4.1%) (ACE*i* of controlled MAEE peptide was 59%) (Figure 1B) and the least in NAEE peptides (1.2%) (ACE*i* of controlled NAEE peptide was 55%) (Figure 1C). The NAEE peptides exhibited more resistance to heat. Wang *et al*. (2017) studied the stability of peptides in mackerel and reported that temperatures >60°C could affect the secondary structure of peptides which are essential to retain biological activity. Also, the reduction obtained in this study was less compared to the reduction (20.4%) in ACE*i* reported for rapeseed protein hydrolysate at 120°C (Wali *et al*., 2017). Extreme heating or sterilization might result in the loss of some of the available peptides and hence, there is some reduction in ACE*i*. However, Singh and Vij (2018) found that ACE*i* peptides from soy were thermally stable at 121°C. In this study, UAEE and MAEE peptides from rohu fish waste showed a 4.2% and 4.1% reduction in ACE*i*, respectively

Table 1. Degree of Hydrolysis, ACE inhibition and Peptide Yield of Peptides obtained by NAEE[1], UAEE[2] and MAEE[3]

	NAEE[1] (%)	UAEE[2] (%)	MAEE[3] (%)
Degree of Hydrolysis (DH)	19.0±1.0[a]	21.5 ±1.3[a]	26.0±1.8[b]
ACE inhibition (ACE*i*)	55.0±1.2[a]	60.0 ±1.4 [b]	59.0±1.1[b]
Peptide Yield (PY)	51.5±0.9[a]	55.0 ±1.2[b]	57.5 ±1.4[b]

[1]Non-Assisted Enzymatic Extraction (NAEE); [2]Ultrasound Assisted Enzymatic Extraction (UAEE); [3]Microwave Assisted Enzymatic Extraction (MAEE)

at 121°C. In corroboration to our study, Akillioğlu and Karakaya (2009) reported a decrease in ACE*i* activity of common beans subjected to heat treatment at 121°C for 15 min and attributed the decrease to a possible change of the protein molecule due to cross bonding (formation of new S-S bonds). Tang (2008) found that heat treatment applied to wild beans (*Phaseolus* spp) has led to the formation of disulfide bridges in the protein molecule. The formation of the S-S bond formation decreases the availability of the SH group. Thus, disulfide bridges stabilize protein conformation and enhance thermodynamic stability (Cui *et al*., 2009). So as the degree of heat induction deepens, the protein tends to unfold initially and then aggregates. Some sulfhydryl groups get oxidized into disulfide bridges and induce protein aggregation, which wraps the enzyme cleavage sites in the protein. Therefore, the formation of new disulfide bridges due to excessive heat induction affects the release of peptides with ACE*i* activity at 121°C.

The retention of ACE*i* activity in tuna cooking juice protein treated at 100°C for 30 min was reported by Hwang (2010), observing only minor changes in peptides. Similarly, soy protein-derived peptides exposed at 100°C for 30 min have exhibited thermal stability and retained ACE*i* activity (Wu and Ding, 2002). The ACE*i* activity of bovine collagen peptide was retained when exposed to relatively low temperatures (20 to 60°C), but a slight reduction in activity was noticed after heating for 2 h at 100°C (Fu *et al*., 2015). The above studies indicate that ACE*i* peptides are relatively stable at 100°C. However, López-Sánchez *et al*. (2016) opined that the loss of stability of ACE*i* activity needs to be assessed under severe treatment conditions, such as sterilization, grilling, and frying, to ensure the expected physiological benefit of food. Extreme heating or sterilization affects the secondary structure of peptides, which had led to a loss of ACE*i* activity in UAEE and MAEE peptides. The effect of heating has less influence on NAEE peptides as they exhibited a higher ACE*i* activity of 55.8% at 121°C. It suggests that NAEE peptides are a suitable ingredient in the development of functional foods involving high heat treatments.

In the case of UAEE and MAEE peptides, exposure to ultrasound and microwave energy has affected the molecular structural stability of peptides (Franca-Oliveira *et al*., 2021). Ultrasound (20 kHz, 4.27±0.71 W and 20% amplitude for 20 min) induced modifications in functional properties of whey protein concentrate, soy protein isolate and egg white protein. These modifications are closely related to molecular changes

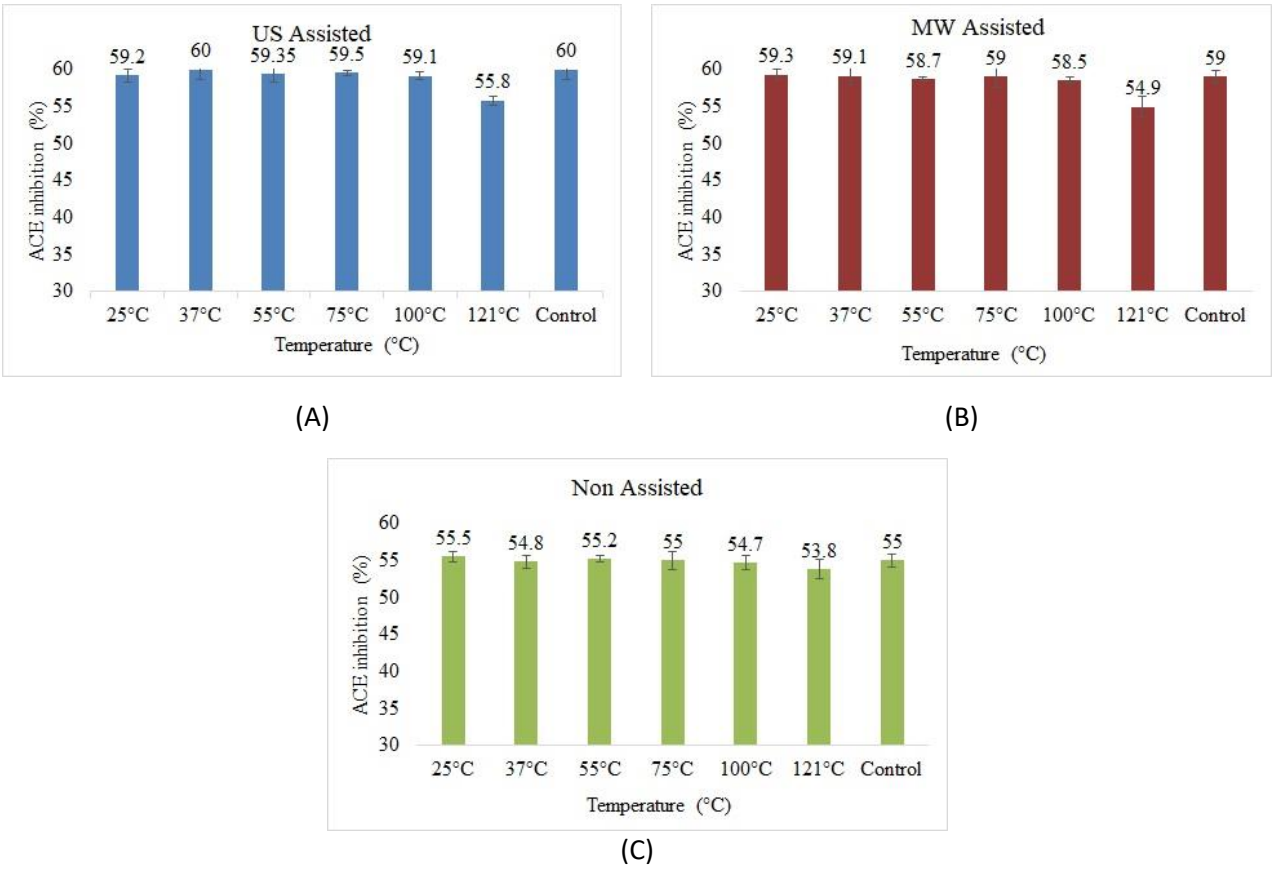

Figure 1. Stability of ACE-inhibitory peptides prepared from rohu fish waste protein to heat treatments. ACE inhibition (%) of peptides obtained by Ultrasound Assisted Enzymatic Extraction (A); Microwave Assisted Enzymatic Extraction (B) and Non-Assisted Enzymatic Extraction (C). Data represent means ± SD (n=3). Data were analyzed by one-way ANOVA. Each Experiment was performed in triplicate. P<0.05 showed statistical significance.

mainly hydrophobicity increase and particle size variation and this change depends on the nature of the protein and its degree of denaturation and aggregation (Arzeni *et al*., 2012). In another study ultrasound treatment (20 kHz; ∼34 W/cm^2; 2 min) significantly ($P<0.05$) reduced aggregate size and hydrodynamic volume of bovine gelatin, fish gelatin and egg white protein, pea protein isolate and soy protein isolate. This reduction was attributed to the hydrodynamic shear forces associated with ultrasonic cavitations. However, no differences were observed in the primary structure molecular weight profile between untreated and ultrasound-treated proteins (O'Sullivan *et al*., 2016). Based on the propagation of pressure oscillations in a liquid medium at the speed of sound, ultrasound-assisted extraction results in the formation, growth, and collapse of microbubbles. The collapse of these microbubbles provokes cell wall breakup, a reduction in particle size, and a mass transfer across cell membranes, allowing the extraction of bioactive peptides from the medium (Franca-Oliveira *et al*., 2021).

In microwave-assisted extraction, microwaves are absorbed by the matrix and converted into thermal energy, which heats the moisture inside the cells. Generating a high pressure on the cells' walls and increasing their porosity microwave facilitates the extraction of bioactive compounds (Franca-Oliveira *et al*., 2021). The change in structural conformation will affect their functional and bioactive properties leading to a reduction in ACE*i* activity. Microwave induces dipolar polarization and electrical conduction and enables rapid heating of polar molecules. On the other hand, ultrasound forms cavitations and their collapse releases high local temperatures and pressures (O'Sullivan *et al*., 2016). The MAEE peptides received energy from the microwave which can penetrate rapidly inside and heat adjacent water molecules within the matrix by dielectric heating. Microwave affects the molecular conformation of proteins due to the formation of inter- and intra-molecular crosslinking leading to aggregation (Hall and Liceaga, 2020). Microwave treatment at 1000W for 5 min has caused cross-linkage between amino acids, affecting *in vitro* protein digestibility; and aggregation increased the percentage of high molecular weight peptides (Xiang *et al*., 2020). Cross-linking of amino acids in the peptides and the resultant aggregation of molecules to form high molecular weight peptides had caused the reduction in ACE*i* activity of MAEE peptides at higher temperatures of 121ºC.

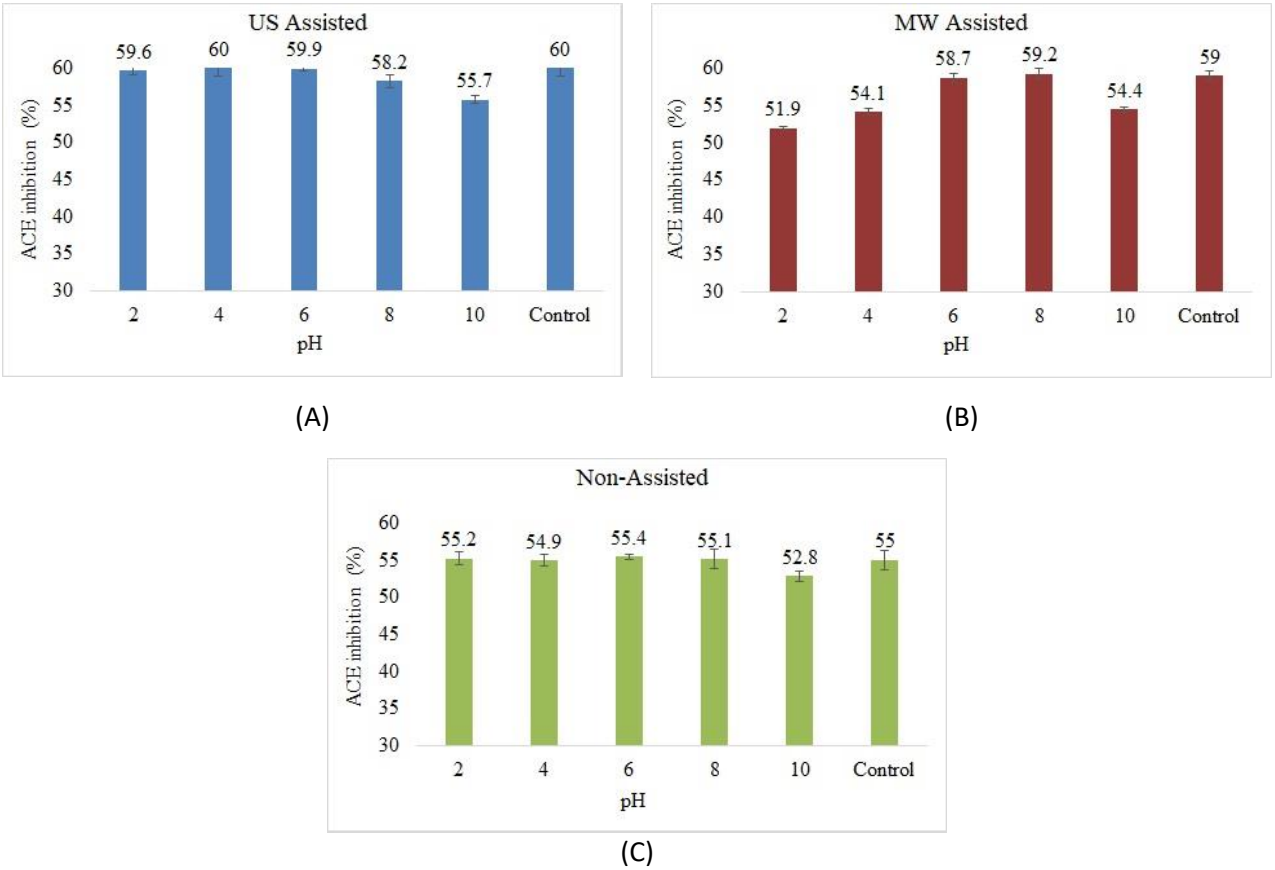

Figure 2. Stability of ACE-inhibitory peptides prepared from rohu fish waste protein to pH treatments. ACE inhibition (%) of peptides obtained by Ultrasound Assisted Enzymatic Extraction (A); Microwave Assisted Enzymatic Extraction (B) and Non-Assisted Enzymatic Extraction (C). Data represent means ± SD (n=3). Data were analyzed by one-way ANOVA. Each Experiment was performed in triplicate. $P<0.05$ showed statistical significance.

Stability to pH

The pH stability of peptides is important to know their fate during the GI digestion process. Peptides, as a constituent in functional food, when entering the digestion system must retain their ACE*i* activity at different stages of digestion. The gastric pH of the stomach is critical for the susceptibility of peptides as it ranges from 2 to 5 and it takes at least 2 h to pass through the stomach after ingestion (Writer, 2020; Seladi-Schulman, 2022). Conversely, sodium bicarbonates secreted by the pancreas neutralize acidic pH once food enters the upper part of the small intestine. The digestive enzymes such as trypsin, chymotrypsin, and other peptidases secreted by the pancreas further aid in digestion at neutral pH (Plessas *et al*., 2017). In our study, peptides retained their ACE*i* activity in acidic pH 2 and near neutral pH 6. The peptides derived from tuna cooking juice were stable in expressing ACE*i* activity at pH 2 and 10 (Hwang 2010). The reduction in ACE*i* activity was high in the MAEE peptides than the UAEE peptides. The decrease in ACE*i* activity observed was similar to that observed with the increase of temperature to 121°C. In rapeseed protein hydrolysate, a decrease in ACE*i* activity from 58% to 53% i.e. 17.35% reduction was observed when pH was increased from 9 to 11 (Wali *et al*., 2017).

The pH triggers denaturation and even hydrolysis of some peptides and thereby affecting their bioactivity (Wu *et al*., 2014). Physicochemical changes of proteins such as denaturation, intermolecular reactions, reaction with sugars, and solubility occur due to pH modification and lead to crosslinking, lysine damage, non-specific cleavage of peptides and amino acids damage (López-Sánchez *et al*., 2016). The pH change also affects the bioactivity by alteration of one or more amino acids (Hwang, 2010). Acid treatment damages glutamine and asparagine whereas alkaline treatment destroys not only cysteine, serine, and threonine, but also produces D-amino acids and lysinoalanine (Kristinsson and Hultin, 2003).

The reduction in ACE*i* activity is attributed to the hydrolysis or denaturation of peptides into inactive form under extremely alkaline conditions (Wali *et al*., 2017). The loss of bioactivity of peptides at higher pH is due to the possible deamination reaction, which might also alter the molecular conformation and structure of peptides (Zhu *et al*., 2014). Therefore, the reduction in

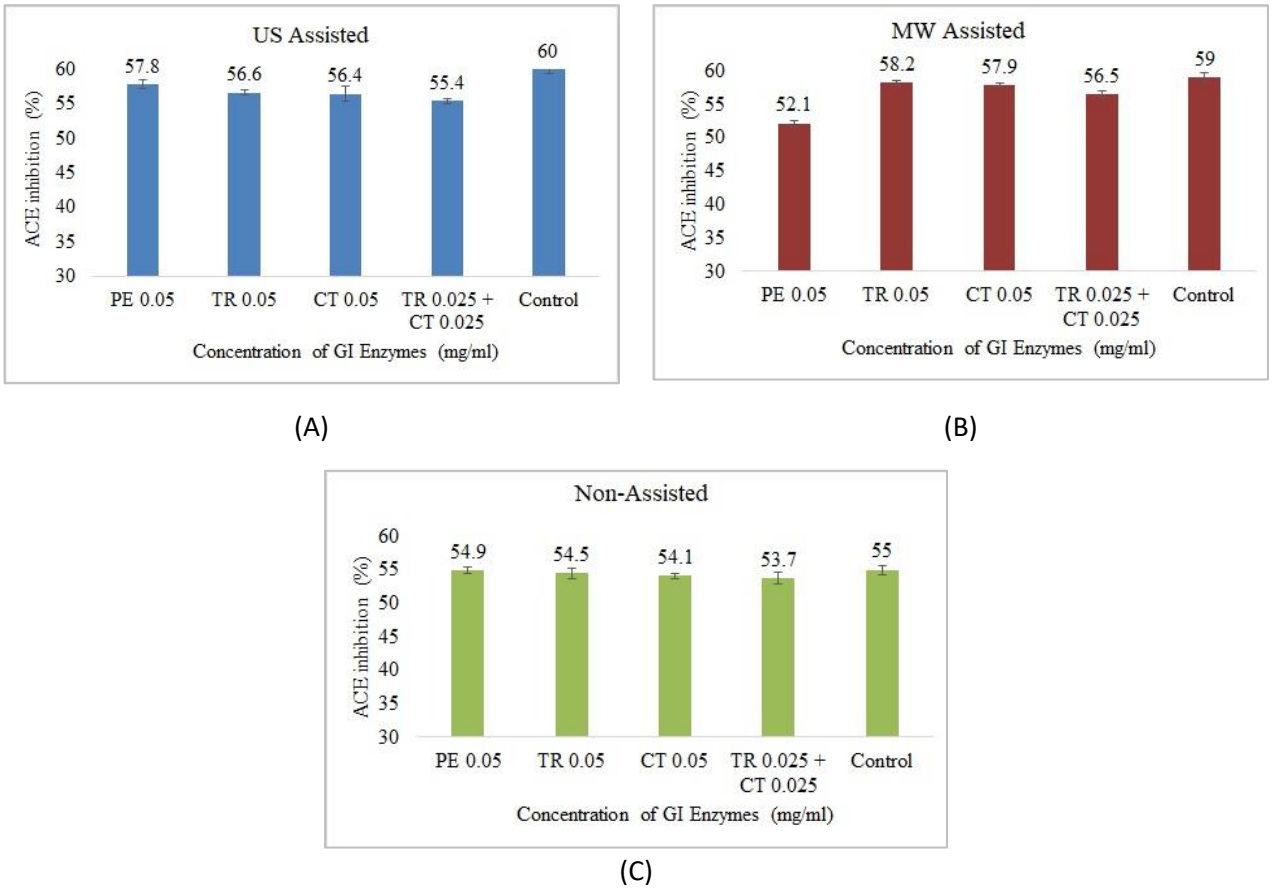

Figure 3. Stability of ACE-inhibitory peptides prepared from rohu fish waste protein to different concentrations of gastrointestinal enzymes. i.e. Pepsin (PE), Trypsin (TR), and Chymotrypsin (CT). ACE inhibition (%) of peptides obtained by Ultrasound Assisted Enzymatic Extraction (A); Microwave Assisted Enzymatic Extraction (B) and Non-Assisted Enzymatic Extraction (C). Data represent means ± SD (n=3). Data were analyzed by one-way ANOVA. Each Experiment was performed in triplicate. P<0.05 showed statistical significance.

ACE*i* activity of peptides derived from rohu fish waste at extreme pH is attributed to possible denaturation or hydrolysis reaction leading to non-specific cleavage of ACE*i* peptides, leading to a loss in bioactivity. High pH affects the tertiary and quaternary structures of proteins at alkaline pH (Deleu *et al*., 2019).

The study showed more reduction in the ACE*i* activity of UAEE and MAEE peptides than the NAEE peptides. The increased destruction of ACE*i* activity has occurred to the action of microwave and ultrasound on the peptides rather than the extreme pH. However, there was no significant difference existed in the ACE*i* activities of UAEE and MAEE peptides subjected to different pH treatments (P>0.05). This indicates that the extent of damage caused by microwave and ultrasound to the peptides are similar.

Stability against Gastrointestinal Enzymes

Besides the effect of pH and temperature on the ACE*i* activity of peptides, their stability to GI enzymes such as pepsin, trypsin, and chymotrypsin is also important to know their bioavailability. *In vitro,* digestion studies were undertaken to examine the effect. The results showed that the UAEE and NAEE peptides retained *in vitro* stability against all GI enzymes. However, MAEE peptides showed less stability with pepsin (0.05%). However, MAEE peptides showed in vitro stability against the treatment with trypsin (0.05%) and chymotrypsin (0.05%). There was no significant (P>0.05) reduction in ACE-inhibition of extracted peptides between treatments when subjected to various enzymatic treatments in UAEE, MAEE and NAEE peptides, which demonstrated that all peptides retained stability against GI enzymes. However, the MAEE peptides exhibited slightly low stability (P>0.05) on digestion with pepsin, as compared to the UAEE and NAEE peptides.

Bougatef *et al*., (2008) observed that a hydrolysate fraction (P4) produced from sardine viscera using alkaline protease showed resistance to *in vitro* digestion by gastrointestinal protease. Also, the ACE*i* activity of soy milk fermented by a *Lactobacillus plantarum* strain C2 was retained before and after treatment with different concentrations of enzymes *viz.* 0.25, 0.50, and 1.0 mg/ml of each trypsin, pepsin, and pancreatin (Singh and Vij, 2018). Toopchan *et al*. (2017) observed a reduction in ACE*i* activity of tilapia mince hydrolysate obtained by *Vibrio halodenitrificans* SK 1-3-7 proteinases in an *in vitro* GI digestion study. Studies on the stability of ACE*i* peptides to different GI enzymes and other microbial enzymes implied that the action of any proteinases within our body does not affect the bioactivity of the resultant peptides, which was also proven from the results of our study, which retained the ACE*i* activity of UAEE and NAEE peptides. On the contrary, ACE*i* peptides produced from spent hen muscle protein using thermolysin were not resistant to simulated GI digestion, as peptides underwent breakdown into smaller peptides (Gu *et al*., 2019).

The molecular size of peptides and enzymes used for hydrolysis plays an important role in influencing ACE*i* activity. The ability of peptides to resist enzymatic hydrolysis depends mainly on their amino acid composition. In general, bioactive peptides encrypted within collagen exhibit greater stability during GI digestion because of the presence of proline (Sun *et al*., 2022). Few reports say that proline-containing peptides are generally more resistant to degradation by digestive enzymes and also, they are better absorbed in the intestine and can reach higher plasma levels after their oral administration (Vermeirssen *et al*., 2004; Sontakke *et al*., 2016). Proline-containing peptides are predominant in collagen and gelatin peptides derived for skin, bones, and frames. As rohu fish waste comprises skin, bones, fins, trimmings, and head, the presence of proline is inevitable. Therefore, peptides derived from rohu fish waste are more stable against digestive enzymes. A slightly lower stability of MAEE peptides upon pepsin digestion is probably because of the modification of secondary structure of protein, including an increase in β-sheets and loss in turns (Dong *et al.,* 2021). The large number of hydrogen bonds in β-sheets reduce the activity of proteases and digestibility (Bai et la., 2016).

Conclusion

The study demonstrated the stability of ACE*i* peptides prepared from rohu fish waste to heat, pH and GI enzymes. UAEE and MAEE peptides retained their ACE*i* activity exhibiting good thermal stability up to 100°C but a reduction at 121°C. The reduction was due to cascading effect of heat in MAEE and UAEE. All peptides retained ACE*i* activity at acidic, neutral pH. and pH 8 (alkaline) and subsequently exhibited a reduction in alkaline pH (8-10) more in MAEE than UAEE and NAEE peptides. Peptides retained ACE*i* activity after enzymatic digestion with GI enzymes *viz.* pepsin, trypsin, and chymotrypsin. The effect of ultrasound and microwave assistance in aiding extraction of peptides had caused a reduction in ACE*i* activity, however not remarkable. The UAEE and NAEE peptides retained *in vitro* stability against all GI enzymes. However, MAEE peptides showed less stability with pepsin (0.05%). The UAEE that exhibited better stability at higher temperatures is recommended over MAEE for extraction of peptides with better stability. Further investigations are required to examine the stability of ACE*i* peptides after incorporation into functional foods. Additional research is also needed to understand the mode of action, clinical efficacy, and structural changes of these peptides.

Ethical Statement

No animals were used in the study which requires approval from the ethical bodies.

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Author Contribution

Data acquisition, calculation, analysis, and preparation of manuscript draft done by V. Kumar; Conception, design of the study, interpretation, and critical revision made by R Jeya Shakila; conception and review by A.U. Muzaddadi; design of the study, editing, and critical review made by G. Jeyasekaran; conceptualization and review by D. Sukumar and P. Padmavathy. All authors have read and agreed to the present version of the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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