Enzymatic treatments of collagen from barramundi (*Lates Calcarifer*) skin for production of bioactive peptides hydrolysates

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Abstract. The utilization of barramundi skin, a by-product of the fish processing industry, as a prospective source of alternative collagen has been explored. To further broaden the prospect of barramundi collagen, its hydrolysate form (CH) was investigated using different enzymatic hydrolysis (alcalase and papain) at various concentration (1%, 2% and 3%). Degree of hydrolysis (DH) of the resulting hydrolysates were determined. The CH were then subjected to physico-chemical characterization including assessment of protein content, FTIR spectra, morphology, solubility and antioxidant properties. Among these, alcalase at a concentration of 1% exhibited notable efficacy, yielding a hydrolysate with DH of 56.5 %, 61.44% ABTS radical scavenging activity and 85% solubility across the pH range studied. Furthermore, both alcalase and papain-derived collagen hydrolysates demonstrated the capacity to disrupt the triple helix structure of crude collagen as observed through FTIR spectroscopy and scanning electron microscopy analysis. These results place collagen obtained from barramundi skin as a potential biotechnological alternative to produce highly potential bioactive peptide-containing hydrolysates.

1 Introduction

Extensive exploration of collagen-derived materials has led to understanding of their versatility in various preparation for food, biomedical, pharmaceutical and cosmetic applications. While collagen has long been recognized for its structural role in tissues, an emerging avenue for maximizing its utility lies in the investigation of its hydrolysed form. Through enzymatic or chemical hydrolysis, collagen is transformed into a soluble state in water, facilitating the release of low molecular weight peptides[1,2]. These peptides, liberated during the hydrolysis process, hold tremendous potential as bioactive compounds, offering a myriad of health-promoting properties and functional benefits in diverse industries[3].

To date, collagen peptides derived from by-products of fish processing is gaining traction due to the safety and religious concern related to conventional collagen sources. Fish collagen hydrolysates treated by enzymatic hydrolysis were reported to possess peptides with antioxidative[4, 5], antimicrobial[6] antihypertensive[7] and anti-freezing[8] properties. The properties of these peptides depend on the hydrolysis conditions employed, which in turn affect their amino acid composition and peptide size[2]. Types of enzymes used, temperature, pH, contact time and enzyme concentration are among the factors affecting their properties.

Barramundi (*Lates calcarifer*), a species of sea bass native to the Indo-West Pacific, has collagen-rich skin that represents a valuable source of the high-quality protein. Collagen from barramundi skin has been previously described[9,10] and it has been discovered that with an improved extraction process, collagen can be recovered effectively from the skin. Hydrolysis of barramundi collagen presents an intriguing avenue for the extraction of bioactive peptides with diverse functional properties, further expanding the collagen application. Alcalase is a serine endopeptidase that exhibits specificity towards peptide bonds adjacent to hydrophobic amino acids [11] while papain, is a cysteine protease known for its broad substrate specificity[12]. Both alcalase and papain were found to give high degree of hydrolysis for collagen hydrolysates from yellowfin tuna[8, 13] and salmon[14, 15]. However, limited information is available for barramundi collagen hydrolysis by alcalase and papain. Thus, the aim of this work was to examine the hydrolysis of collagen obtained from barramundi skin using varying concentrations of papain and alcalase, respectively. The resulting collagen hydrolysates were then evaluated for their physico-chemical properties and antioxidative capacity.

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2 Materials and methods

2.1 Materials

Barramundi (*Lates calcarifer*) skins were supplied by a local fish processing premise at Johor Bahru, Malaysia. The skins were cleaned and cut into smaller size (0.5 cm x 0.5). Prior to collagen extraction, the skins were stored at -20 °C.

2.2 Collagen hydrolysate preparation

Collagen was first extracted from barramundi skin as previously reported by Razali et al.[10]. Barramundi collagen (0.25 g) was mixed in 50 ml of 0.2125 M phosphate buffer prior to hydrolysis with alcalase (A1, A2, A3) and papain (P1, P2, P3) at varied concentrations (1%, 2%, 3% E/S), respectively. The enzyme hydrolysis was conducted for 3 h with continuous stirring under the conditions as specified in Table 1[16]. The mixture was heated for 15 min at 90 °C to stop the enzyme reaction. Supernatant was collected after centrifugation at 10,000 g for 30 min at 4 °C. The obtained collagen hydrolysates (CH) were freeze-dried kept at -20°C prior to analysis.

Table 1. Enzymatic hydrolysis conditions of barramundi collagen

Enzyme	Optimum Conditions		
	Time (h)	Temp (°C)	pH
Alcalase	3	50	8
Papain	3	37	6.5

2.3 Degree of hydrolysis

The efficiency of collagen hydrolysis by the different enzymes at different concentration was evaluated based on the degree of hydrolysis (%) (DH). A glycine standard was used to determine the α-amino acid content released [17].

2.4 Protein recovery content

The protein content of collagen hydrolysates was assayed using Bradford method using a BSA (bovine serum albumin) standard calibration curve [18].

2.5 Solubility

Solubility of collagen hydrolysates at different pH conditions was evaluated as mentioned by Zamorano et al[6]. Solubility of CH were calculated based on protein content as measured using Bradford method according to the following equation:

$$\textbf{Solubility}\ (\%) = \frac{A}{B} \times 100\% \qquad (1)$$

where A is the protein content measured from supernatant, and B is the protein content of the collagen hydrolysate solution.

2.6 Antioxidant activity

The $ABTS^+$ radical scavenging activity of CH from barramundi collagen was evaluated according to León-López et al.[19].

2.7 Fourier transform infrared spectroscopy (FTIR)

Secondary structure of collagen hydrolysates was determined as described by Liao et al.[19]. Lyophilised collagen hydrolysates (0.5 mg) were grounded under dried conditions with 100 mg of KBr. The reading were measured at spectra ranging from 500 to 4000 cm^{-1} with a resolution of 2 cm^{-1} using FTIR spectrometer model Nicolet iS10 (Thermo Scientific, Madison, WI, USA). The measurements were contrasted with the background spectrum of an uncontaminated empty cell at ambient temperature. FTIR spectra of collagen from barramundi skin was also evaluated for comparison.

2.8 Scanning electron microscopy (SEM)

Morphology structure of collagen hydrolysate from barramundi skin was observed using scanning electron microscopy (SEM) (Quanta FEG 650, Edinburgh, United Kingdom). The sample was placed on a stub and coated with platinum using sputter coater (Q150T Plus, East Sussex, United Kingdom) prior to measurement. The microstructure of collagen hydrolysate from barramundi skin hydrolysed by the different enzymes was evaluated.

2.9 Statistical analysis

Data collected are reported as a mean ± SD of triplicate measurements. One-way analysis of variance was conducted using SPSS software (version 27.0, SPSS Inc., Chicago, IL, USA). The significance of the variances between the means was evaluated using Duncan's multiple range test.

3 Results and discussion

3.1 Degree of hydrolysis

The degree of hydrolysis (DH) of collagen from barramundi skin after alcalase and papain treatments at different concentrations is as shown in Figure 1. Alcalase and papain exhibited different capability to hydrolysed collagen from barramundi skin, with concentration of the enzyme play a significant role. The highest DH was observed for A1 (56 %) whereas the lowest value was found in A3 (39 %). It was discovered that alcalase at lower concentrations was effective enough to hydrolyze barramundi collagen, as opposed to papain, which had the highest DH at 3% concentration.

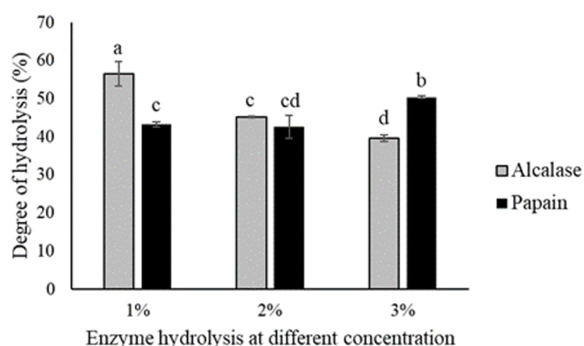

Fig. 1. Degree of hydrolysis of collagen hydrolysates from barramundi skin hydrolysed by alcalase and papain at different concentration. Values represent the mean ± SD (n=3). Different superscript letters indicate statistically significant differences ($p<0.05$).

The DH reported in this study was significantly higher than that reported for yellowfin tuna skin using alcalase hydrolysis at 0.034 AU/g of enzyme concentration[8] and papain hydrolysis of CH from sturgeon fish skin at 1%[16]. While papain displayed a wider hydrolytic activity toward other proteins remained in the skin matrix following pretreatment, alcalase may more precisely hydrolyse collagenous materials in the skin, which may account for increased DH [4].

3.2 Protein content

Enhanced protein degradation is indicated by the collagen hydrolysate's low protein recovery concentration [20]. The protein content of collagen hydrolysate from barramundi skin extracted using alcalase and papain at different enzyme concentration are shown in Figure 2. In contrast to DH, the protein content displayed a trend of lower values attained with alcalase treatment. CH from barramundi skin treated with the different enzyme concentration in this study has a lower protein content than CH from sheepskin hydrolyzed with trypsin at 2% [21].

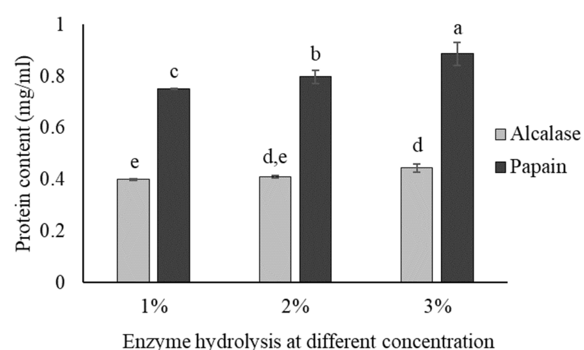

Fig. 2. Protein content of collagen hydrolysates from barramundi skin hydrolysed by alcalase and papain at different concentration. Values represent the mean ± SD (n=3). Different superscript letters indicate statistically significant differences ($p<0.05$).

3.3 Solubility

The solubility of CH derived from varying alcalase and papain hydrolysis concentrations at different pH (4, 7, and 10) is depicted in Figure 3. CH produced using both papain or alcalase displayed high solubility (>85%) throughout all the pH range under investigation. Following hydrolysis, collagen molecules break down into smaller peptides. This cleavage of peptide bonds within the collagen structure releases polar carboxyl and amide groups, which have a strong affinity for interacting with water, resulting in enhanced solubility[22]. The results were in line with those reported for gutted silver carp collagen hydrolysate[23]

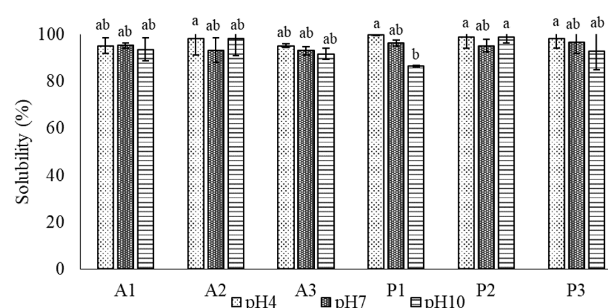

Fig. 3. Solubility of collagen hydrolysates from barramundi skin hydrolysed by alcalase and papain at different concentration at pH 4,7 and 10. A1: alcalase 1%, A2: alcalase 2%, A3: alcalase 3%, P1: papain 1%, P2: papain 2%, P3: papain 3%. Values represent the mean ± SD (n=3). Different superscript letters indicate statistically significant differences ($p<0.05$).

3.4 ABTS+ free radical scavenging activity

The antioxidant properties of CH as described by $ABTS^+$ radical scavenging activity for papain and alcalase at different concentrations are as shown in Figure 4.

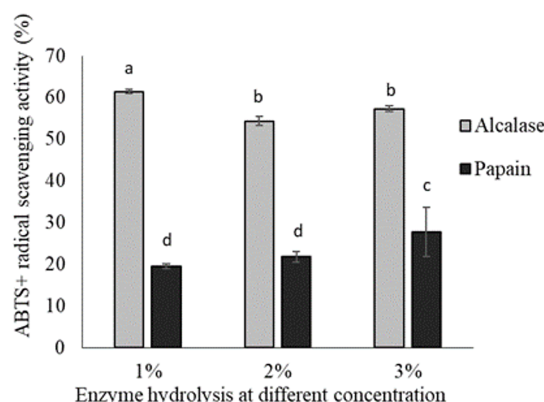

Fig. 4. $ABTS^+$ radical scavenging activity of collagen hydrolysates from barramundi skin hydrolysed by alcalase and papain at different concentration. Values represent the mean ± SD (n=3). Different superscript letters indicate statistically significant differences ($p<0.05$).

$ABTS^+$ radical scavenging activity for CH obtained by alcalase was significantly higher that papain at all

determined concentrations. The antioxidant activity of barramundi CH is in tandem with the DH where 1 % of alcalase showed the highest. The antioxidant activity of peptides is often influenced by their amino acid composition, the amount of free amino acids, the degree of hydrolysis, and their molecular weight[20,24]. Specifically, low molecular weight hydrolysates exhibit superior antioxidant properties in comparison to high molecular weight hydrolysates[20].

The $ABTS^+$ radical scavenging activity of barramundi CH found in this study was lower than that reported for CH from milkfish scale using pepsin hydrolysis at 1 %. [25]. Interestingly, the highest $ABTS^+$ radical scavenging activity observed for A1 was comparable to that of low molecular weight peptides (<3 kDa) derived from jumbo squid muscle. [26]. Alcalase hydrolysis of barramundi collagen may release more peptides of low molecular weight.

3.5 Fourier transform infrared spectroscopy (FTIR)

The IR spectra for collagen and collagen hydrolysates from barramundi skin extracted using different enzymes are as shown in Figure 5. The N-H stretching vibration related to Amide A absorption spectrum emerged between 3000 and 3500 cm^{-1} [27]. The Amide A peaks of crude collagen, collagen hydrolysate by alcalase and papain were identified at 3293.01 cm^{-1}, 3521.59 cm^{-1} and 3483.44 cm^{-1} respectively. There is a difference in the chemical environment of the peptide bond between crude protein and collagen hydrolysate, revealed an intensified peak that changed to a higher wave number in the collagen hydrolysate extracted.

The distinctive absorption bands of crude collagen, CH-alcalase, and CH-papain, respectively, were visible in the amide II region at 1544.86, 1537.54, and 1548.14 cm^{-1}. When compared to crude collagen, the amplitude of CH-alcalase and CH-papain amide II were reduced, indicating that N-H was more involved in bonding with the neighbouring α-chains[28]. Due to the high degree of disorder in the molecular structure, the change of the α-helical structure into a random coil structure happened concurrently with the enzyme process. The modifications were related to the loss of the triple-helix state caused by the denaturation of collagen into collagen hydrolysate. This can be seen by moderate intensity peaks at shorter wavelength, between 800 and 1200 cm^{-1}, which were not present in the spectrum of the crude collagen sample.

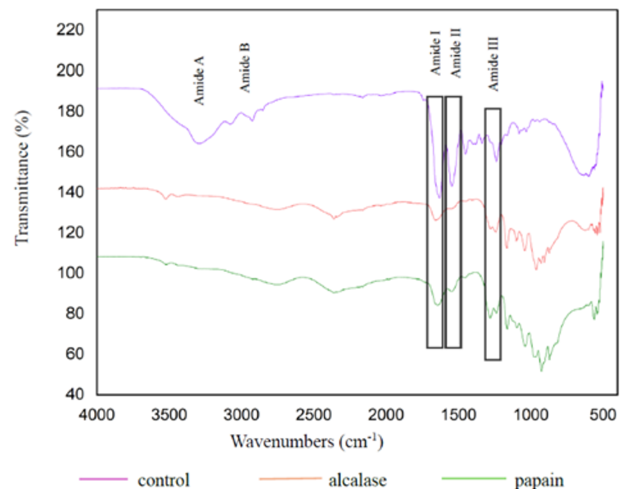

Fig. 5. FTIR spectra of the collagen and collagen hydrolysate from barramundi skin

3.6 Scanning electron microscopy

SEM micrographs of CH from barramundi skin are displayed in Figure 6. The results demonstrate that hydrolysis disrupt the integrity of collagen particles, resulting in irregularities in morphology. A similar observation was found for collagen from sheepskin that had been hydrolysed with trypsin, yielding a porous and spongy hydrolysate[21]. It was suggested that more porous structure indicated that the collagen structure is more exposed and related to the presence of lower molecular weight peptides [29].

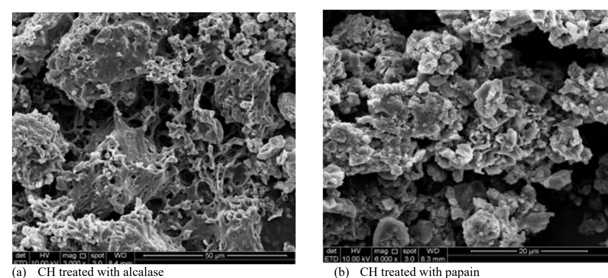

Fig. 6. SEM images of collagen hydrolysates from barramundi skin prepared by (a) alcalase and (b) papain.

4 Conclusion

Barramundi collagen was successfully hydrolysed using alcalase and papain at different concentrations with varying properties. Alcalase at lower level was effective to hydrolyse barramundi collagen and liberated peptides with higher antioxidant activity as compared to papain. The different enzymatic hydrolysis settings disrupted collagen structure in different ways, which can be controlled to generate hydrolysate exhibiting targeted properties.

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