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Assay of proteolytic activity of *Bacillus Subtilis* for collagen isolation from Snakehead Fish (*Channa striata*) scales

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Abstract

The collagen source could be derived from fish scales like snakehead fish (*Channa striata*). One of the possible methods can be used enzymatic methods. The objective of this research was to isolate and characterize the collagen from *C. striata* scale using *B. subtilis* protease. This study was an experimental method utilizing the protease enzyme produced by *B. subtilis*. The proteolytic activity of *B. subtilis* was seen from the ability of the bacteria to produce a clear zone on skim-casein agar and inoculated into tryptic soy broth (TSB) as a submerged medium to produce protease. The collagen isolation using *B. subtilis* protease (crude and freeze-dried enzyme) with ratios 1:1, 1:10, 1:100, and 1:1000 and incubation for 6, 12, 18, and 24 hours. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the molecular weight (MW) of collagen proteins, protein determination by Bradford methods, and morphology was observed by scanning electron microscopy (SEM). All data were analyzed using using SPSS.16 software for windows. The highest collagen was at 12 hours (1:100) incubation using freeze-dried enzymes resulting in a mass of 0.10 grams and a protein content of 3.17 g/ml ($p<0.05$). SDS-PAGE and SEM visualization showed the collagen might be classified as type I collagen consisting of two chains (α1 and α2) with molecular weights approaching 118.03 kDa and 112.20 kDa. Collagen from Channa striata scales can be isolated enzymatically using a protease produced by B. subtilis and can be characterized as type 1 collagen.

Keywords: *Bacillus subtilis, Channa striata*, collagen, protease, scale

1. Introduction

Collagen is the fibrous protein that contributes to the unique physiological functions of connective tissues in skin, tendons, bones, cartilage, and others (1). It is also found in the interstitial tissue of all parenchymal organs, where they contribute to the stability of tissues and organs and maintain their structural integrity (2). Collagen is synthesized and secreted by mesenchymal and epithelial cells. Mesenchymal cells and their derivatives such as fibroblasts, osteoblast, odontoblast, chondroblasts, and cementoblasts are the source of collagen. A basic structural unit of collagen is tropocollagen - α-helix left-handed molecule with transverse bands repeating every 64–67 nm and composed of 3 bonds of collagen monomers (*triple-helix)*. *Triple helix* collagen is highly resistant to proteolytic attacks such as matrix metalloproteinases (MMP) (2).

Collagen is a long-chain protein composed of the amino acid alanine, arginine, lysine, glycine, proline, hydroxyproline, and hydroxylysine, but mostly consist of glycine (33%), proline, and hydroxyproline (22%) (3). The structure of Gly-Pro-Hyp amino acid sequence has been given in the Fig. 1 (4). The amino acid sequence is composed of collagen monomers with glycine in every three residues (5). The sequence of the peptide is (Pro-Lys-Gly) (Pro-Hyp-Gly) (Asp-Hyp- Gly). Furthermore, the characteristic feature of collagen is the presence of the amino acid hydroxyproline. The glycine-X-Y arrangement is continuously repeated, with X and Y being proline and hydroxyproline (5, 6).

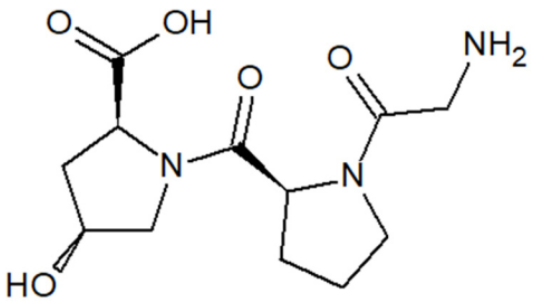

Fig. 1. The structure of Gly-Pro-Hyp amino acid sequence (4)

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Collagen is composed of connective tissue extracellular matrix (ECM) consisting of fibrillar collagen, forming collagen fibrils (3). The function of collagen fibrils is to produce structures with very high tensile strength without the ability to stretch. Collagen fibrils can support at least 10,000 times their weight. Collagen fibers are generally white and opaque and look like typical and repeating transverse lines when observed with scanning electron microscopy (SEM) (1). Collagen has been applied in food manufacturing, pharmaceutical, cosmetic, biomedical materials, and clinical fields (7). The most abundant primary collagens are types I, II, and III for health solution. It has low immunogenicity and is minimally rejected in the human body. It is widely used to help blood clotting, healing, and tissue remodeling. The major, most abundant collagens are types I, II, and III which form the structural fibrils of tissues, while the others only take part in the association of these fibrils with others (1).

Collagen is commonly isolated from plant-based and animal source such as skins and bones of mammals, especially bovine and porcine (1). Among these animal sources, bovine collagen is commonly used for extra-oral and burns wound healing. Porcine collagen is unacceptable for some religions, for example, Judaism and Islam. Moreover, bovine collagen has a risk of contamination from bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), foot-and-mouth disease (FMD), protein misfolding, and allergenicity (8). Consequently, much attention has been paid to the alternative sources of collagen such as bones, skin, fins, and scales of fresh or saltwater fishes (9,10). Fish collagens were considered good alternative sources because there is no risk of disease transmission, and they have a high yield (1). Fish scales are mainly composed of hydroxyapatite $Ca_{10}(OH)_2(PO_4)_6$ and type I collagen fiber with a lower denaturation temperature than the collagen from porcine (11). The source of fish scale collagen such as black drum, sheep's head sea bream, Red-seabream, Red Tilapia, sardine, Japanese sea-bass, skipjack tuna, yellow sea bream, and horse mackerel have been reported. Furthermore, there is limited information of collagen isolation from snakehead fish scales. Snakehead fish (*Channa striata*) is a freshwater fish from the *Channidae* family. This fish is a predator for other small fish and tadpole on fresh water ecosystem. In, Indonesia snakehead fish can be found throughout Indonesia, especially in Sulawesi and Papua (12).

The collagen isolation process needs to be noticed in order to get applicable collagen in society. Collagen can be obtained from the hydrolysis of fish scales with chemicals or enzymatic. Enzymatic hydrolysis using a biological process such as enzyme produced by organism is more promising due to generating less waste and may reduce the processing time (13). Proteases were the most important groups of extracellular enzymes which produced by animals, plants and microorganisms (14). Furthermore, microbial proteases have been exploited in the leather industries in many ways because their ability to produce protease in large proportions. A large proportion of the proteases are derived from Bacillus strains (15), such as *B. subtilis.* Therefore, the objective of this research was to isolate and characterize the collagen from *Channa striata* scale using *B. subtilis* protease

2. Materials and methods

2.1. Research design

This research was an experimental study with a completely randomized block design including incubation time (6, 12, 18, and 24 hours), the type of enzyme (crude and freeze-dried enzyme), and the ratio of the enzyme and substrate (1:1, 1:10, 1:100, and 1:1,000). In each group, 3 repetitions were carried out so the total number of samples was 96. This research was conducted from December 2014 to May 2015 at the molecular biology laboratory, animal anatomy and physiology laboratory and microbiology laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, University of Brawijaya.

2.2. *Bacillus subtilis* protease production

B. subtilis was grown in skim-casein agar containing (g/L) 5 caseins; 2.5 yeast extract; 28 skims; and 15 bacteria agar and incubated at 37°C for 24 hours. After 24 h incubation, plates were observed for each clear zone around the growth of the organism. If a clear zone is seen, it indicates that *B. subtilis* has the ability to degrade protein through the presence of proteases. Then, *B. subtilis* was inoculated into tryptic soy broth (TSB) as an optimal submerged medium containing (g/L) 170 triptone; 30 soy peptones; 25 dextrose; 50 NaCl; and 25 K_2HPO_4 and cultured in a shaker incubator (180 rpm) at 37°C for 48 hours (16). The whole fermented broth was centrifuged at 10,000 rpm at 4°C for 10 minutes and the supernatant was obtained. Crude enzymes from the supernatant were subjected to further research and were continuously lyophilized by freeze-drying machines as a source of proteases.

2.3. Protease assay

The protease activity from crude and freeze-dried enzyme from *B. subtilis* was measured using casein as a substrate. A mixture of 200 µl (500 ppm) of casein (in 300 ml phosphate buffer pH 7), and 100 µl protease (crude and freeze-dried enzyme) were incubated in an incubator at 37°C for 60 minutes. After that, the enzyme reaction was terminated by the addition of 400 µl of 4% (w/v) trichloroacetic acid (TCA) and was kept at room temperature for 30 minutes. Then, the mixture was centrifuged to separate the unreacted casein at 4,000 rpm for 10 minutes. The supernatant was diluted in 5 times phosphate buffer and measured the absorbance at 275 nm (16). The protease activity profile of the supernatant from *B. subtilis* on TSB media and measured by formula (Formula 1). One unit of protease is defined as the amount of enzyme that releases 1 µg of tyrosine per ml per minute under the standard conditions of supernatant solution (17).

$$\frac{[C]}{(Mr*t)} \quad \frac{x}{E} \quad H \quad x \quad Df$$

Formula 1. [C], concentration (U/ml); Mr, molecular weight of tyrosine (181.19); t, time (minute); H, total volume (ml); E, enzyme volume (ml); Df, dilution factor

2.4. Enzymatic isolation of collagen from snakehead fish scale

All preparation procedures were performed at 4°C. The powder of Kanjilo or snakehead fish scale was obtained from Makassar, South Sulawesi, and soaked in 100 ml of 0.5 M acetic acid for 24 hours and centrifuged at 4,500 rpm for 15 minutes. *B. subtilis* protease added into supernatant comparing of (v/v) 1:1, 1;10, 1:100 and 1:1,000 then incubated for 6, 12, 18, and 24 hours. The mixture was centrifuged at 4,500 rpm for 30 minutes to separate and precipitate the fibrous collagen. Pellets were mixed in 0.5 M acetic acid (1:9) and dialyzed using a cellophane membrane in 0.1 M acetic acid and deionized water to get pepsin solubilized collagen (PSC) (18).

2.5. Protein determination of collagen from snakehead fish scale

Total protein content was measured by Bradford methods using bovine serum albumin (BSA) as a standard protein. Collagen samples were taken100 µl and added 1 ml of diluted PRO-MEASURE solution. Then incubated for 2 minutes at room temperature and measured the absorbance at 595 nm (iNtRon Biotechnology, Inc) (19).

2.6. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed by Laemli methods. 10 µl collagen samples were loaded onto polyacrylamide gel made of 12,5% separating gel and 3 % stacking gel. After electrophoresis, each gel was stained by Coomassie Brilliant Blue (CBB) R-250 for 15 minutes and destained for 30 minutes (20).

2.7. Morphology analysis

The morphology of collagen was observed by Hitachi High-Technologies scanning electron microscopy (HITACHI-TM3000 SEM).

2.8. Statistical analysis

The means of each data and standard deviation were calculated using Microsoft Excel. Furthermore, data from each group, including incubation time (6, 12, 18, and 24 hours), type of enzyme (crude and freeze-dried enzyme), and the ratio of enzyme and substrate 1:1, 1:10, 1:100, and 1:1,000, were analyzed using factorial test or completely randomized block design using SPSS.16 software for windows

3. Results

In this study, the extraction of collagen from snakehead fish scales supernatant was carried out using a protease enzyme from *B. subtilis.* The enzyme treatment was based on the ratio enzyme volume (µl) compared to snakehead fish scales supernatant volume (µl), which included 1:1, 1:10, 1:100, and 1:1,000.

Fig. 2 shows that at 1:1,000, the enzyme still works optimally and produces collagen mass mostly at 12 hours of incubation. The highest mass of collagen produced by crude enzyme was 0.34 gram, while for the freeze-dried enzyme, the collagen mass tended to increase following the incubation time. Furthermore, when compared at 1:1, 1:10, and 1:100 at 12 hours incubation, the highest collagen mass was found at 1:100 for crude enzymes and freeze-dried enzymes, which were 0.15 gram and 0.10 gram, respectively.

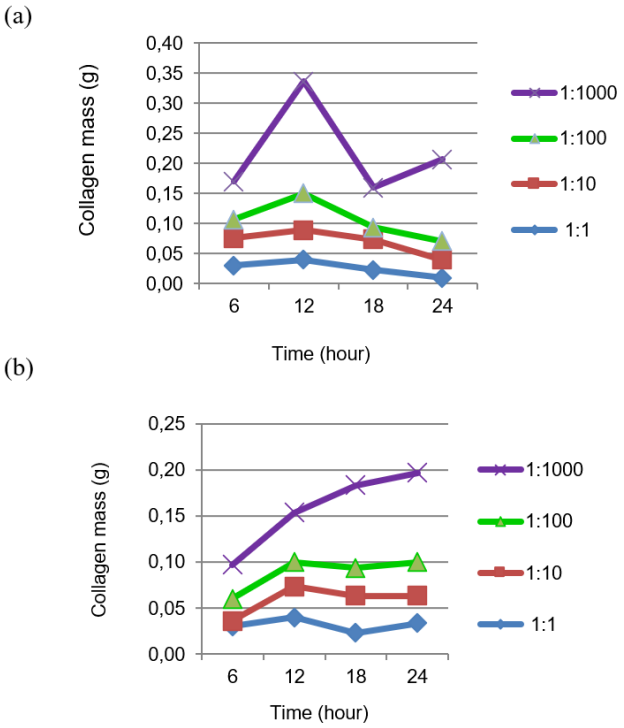


Fig. 2. Collagen mass produced by snakehead fish scales isolation with crude enzyme (a) and freeze-dried enzyme (b)

3.1. The protein content of snakehead fish scale collagen isolated using the *B. subtilis* protease

The protein content of snakehead fish scale collagen was determined by the Bradford method, which measured the total protein concentration in a solution by colorimetry.

Fig. 3 shows the protein content of snakehead fish scales collagen isolated using the *B. subtilis* protease, both crude enzyme (a) and freeze-dried enzyme (b). In this research using 1:1,000, the crude enzyme produced the highest levels of collagen protein at 12 hours of incubation (2.47 µg/ml), while the freeze-dried enzyme using 1:1,000 produced relatively lower that was 1.96 µg/ml at 24 hours of incubation. Moreover, the highest protein content of collagen was produced by 1:100 at 12 hours incubation using freeze-dried enzyme as much 3.17 (µg/ml) ($p<0.05$).

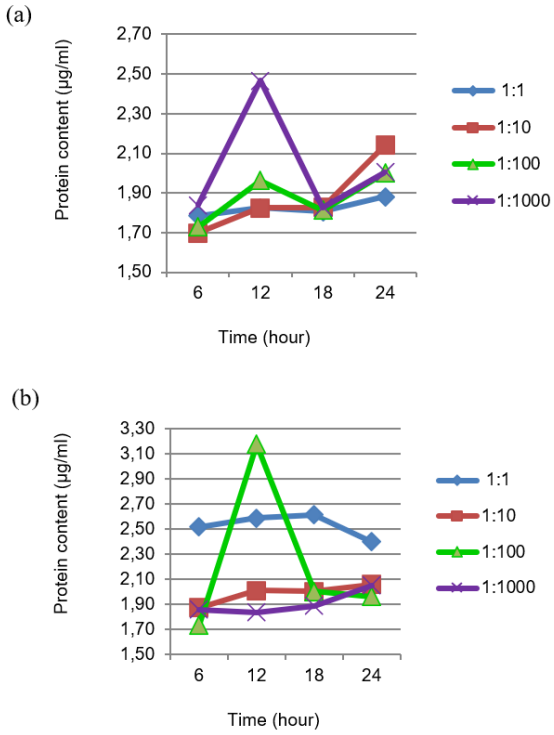


Fig. 3. Total protein content produced by snakehead fish scales isolation with crude enzyme (a) and freeze-dried enzyme (b)

3.2. Molecular weight of collagen from snakehead fish

The SDS-PAGE analysis of PSC from snakehead fish scale was shown in Fig. 4.

The protein banding pattern produced by SDS-PAGE in this study showed that the collagen residues from snakehead fish scales were composed of α- and β-chain that appeared to coincide. Collagen from the snakehead fish scale consists of two α-chains (α1 and α2) with the molecular weight of approximately 118.03 kDa and 112.20 kDa, respectively, and β chain with a molecular weight 137.40 kDa.

3.3. Snakehead fish collagen visualization

The fibrils formation profile shown in Fig. 5 from SEM visualization presented the collagen fibril morphology. The morphology showed random coil structure and obvious fibril networks with the rough membranous structure for the collagen membrane.

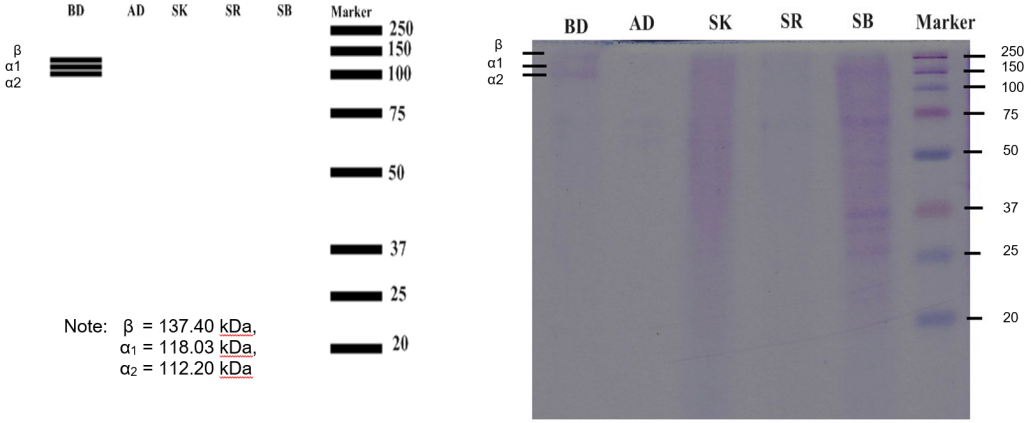
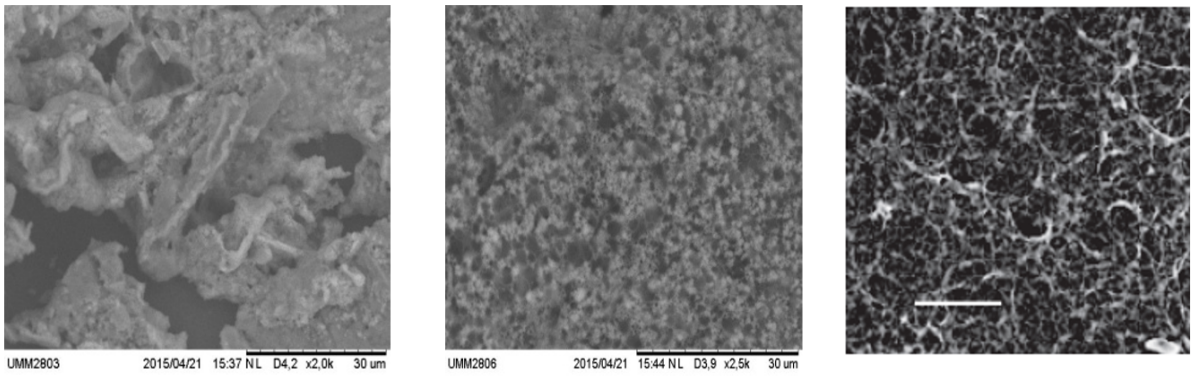


Fig. 4. The molecular weight of collagen type 1 from snakehead fish scales. *BD, before dialysis; AD, after dialysis; SK, sisik kering (dry scales); SB, sisik basah (wet scales)*



(a) (b) (c)

Fig. 5. SEM visualization of collagen from snakehead fish scales with mag. 2000x (a), 2500x (b), and collagen from bovine Bar 5 µm (30) (c)

4. Discussion

This research is a continuation of previous research carried out by the author (16). Relative protease activity was shown by clear zones around the growth of the *B. subtilis* colony on skim-casein agar plates. It was explained that the *B. subtilis* has the ability to degrade protein, which breaks the peptide bond CO-NH into free amino acids. Skim-casein agar was the media that contained skim milk and casein. The media had the function of inducing alkaline protease synthesis by bacteria. The protease activity of *B. subtilis* was influenced by temperature and incubation time. The increase of temperature which is more than optimum temperature will affect to the conformational changes of the substrate so that the substrate is inhibited and reduces enzyme activity. The protease activity of *B. subtilis* can be stable at temperatures between 30°C to 60°C and optimum at 37°C (21). Moreover, the optimum incubation time around 48 hours (22). Measurements of protease activity were interpolated against the standard tyrosine curve. Tyrosine standard curve was made with several variations of tyrosine concentration (10-100 ppm) and measured with absorbance of 275 nm. The absorbance value is the maximum wavelength for UV absorption by aromatic amino acids such as tyrosine, tryptophan, and phenylalanine (17).

Samples of crude enzyme and freeze-dried enzyme from *B. subtilis* were measured for their protease activity using casein as a substrate. The amount of protease activity was determined based on the amount of tyrosine produced from the hydrolysis of casein at a wavelength of 275 nm. One unit of enzyme activity is expressed as the volume (ml) of enzyme required to produce 1 mg of tyrosine per minute from casein substrate (Formula 1). The result showed that protease activity from crude enzyme and freeze-dried enzyme *B. subtilis* was significantly different ($p<0.05$). The crude enzyme produced 0,134 U/ml, while freeze-dried enzyme produced 0.106 U/ml. The protease activity was decreased in freeze-dried enzyme because the lyophilized or freeze-drying process induce the inactivation of the enzyme and was also reduced by the presence of a proteinaceous additive, such as sugar molecules that exists naturally (23). Furthermore, the stability of the enzyme during the freezing and freeze-drying process is affected by concentration (23), while in this research, the enzyme concentration was not measured.

Collagen extraction was carried out through a series of complex procedures, starting with the collection of raw materials, pretreatment, extraction, and purification. The two most common extraction methods are acid-soluble collagen (ASC) and pepsin-soluble collagen (PSC) (13). Based on the research of Schmidt (2016), it is known that PSC is the most ideal extraction method because the enzyme has a greater reaction selectivity. In this study, the extraction of collagen dissolved in the snakehead fish scales supernatant was carried out using a protease enzyme from *B. subtilis* which acts like pepsin. The enzyme treatment was based on the ratio (enzyme volume: snakehead fish scales supernatant volume), which included 1:1, 1:10, 1:100, and 1:1,000.

Collagen mass produced from snakehead fish scale influenced by time incubation, type of enzyme (crude or freeze-dried enzyme from *B. subtilis*), and ratio hydrolyzed sample volume from snakehead fish scales supernatant. Fig. 2 shows the different volumes of the substrate (1:1, 10, 1:100 dan 1:1,000) from snakehead fish scales supernatant can be hydrolyzed by the enzyme from *B. subtilis* as much as 1µl ($p<0.05$). Moreover, the figure shows that at 1:1000, the enzyme still works optimally and produces collagen mass mostly at 12 hours of incubation. The highest mass of collagen produced by crude enzyme was 0.34 grams, while for the freeze-dried enzyme, the collagen mass tended to increase following the incubation time. Furthermore, when compared at 1:1, 1:10, and 1:100 at 12 hours incubation, the highest collagen mass was found at 1:100 for crude enzymes and freeze-dried enzymes, which were 0.15 gram and 0.10 gram, respectively.

The extraction method in this study was in line with Matmaroh et al. (2011), which stated that collagen extraction from sharp nose stingray fish scales was more soluble in the presence of the pepsin (PSC) compared to the extraction method using only 0.5 M acetic acid (ASC). Pepsin is a type of protease enzyme. This enzyme plays a role in breaking down the collagen cross-links in the telopeptide region (18). Many studies have been carried out on collagen extraction using the PSC method, such as the cartilage of the brown-banded bamboo shark with the collagen yield of 9.59% (24), the jelly fish as much as 60% (25), the swim bladder of yellow-fin tuna as much as 12.10% (26), and so on. This shows that the use of pepsin in the hydrolysis of collagen can break the polypeptide chain in certain areas without destroying the integrity of the collagen triple helix structure, then resulting in higher PSC (27).

The protein content of snakehead fish scale collagen was determined by the Bradford method, which measured the total protein concentration in a solution by colorimetry. The composition of Bradford's solution was Coomassie brilliant blue (CBB) which was acidic and could bind to the proteins, then changed the solution color to blue. Fig. 3 shows the protein content of snakehead fish scales collagen isolated using the *B. subtilis* protease, both crude enzyme (a) and freeze-dried enzyme (b). The protein content measurement was carried out on the residual mass of collagen obtained from isolation method (fig. 2). However, the amount of residual collagen mass was not influence to the measurement of protein content ($p>0.05$). This could be seen in the comparison of 1:1,000, which produced the highest collagen residue mass (fig. 2) but produced fluctuating protein content (fig. 3). In this research using 1:1,000, the crude enzyme produced the highest levels of collagen protein at 12 hours of incubation (2.47 µg/ml), while the freeze-dried enzyme produced relatively lower that was 1.96 µg/ml at 24 hours of incubation. This happens because an

enzyme can become unstable in the freeze-thawing and freeze-drying process before being used (28). Proteins are also thought to be more susceptible to denaturation under various stress conditions (23). Moreover, fig. 2 and 3 shows the highest collagen mass and protein content produced by 1:100 at 12 hours incubation using freeze dried enzyme. The quantities of collagen mass and protein content were produced, approximating 0.10 gram and 3.17 (μg/ml) ($p<0.05$).

The protein banding pattern produced by SDS-PAGE in this study showed that the collagen residues from snakehead fish scales were composed of α- and β-chain that appeared to coincide. It is caused by the amino acid composition of α- and β-chain is not much different and has almost the same relative molecular weight. This figure also showed that the molecular weight of collagen could be classified as type 1. Collagen type I was the dominant type of collagen that used in cosmetics properties because the human skin was mainly built by type I, III, and V collagen. Moreover, collagen can accelerate wound healing and tissue regeneration. This collagen was composed of two different α-chain (α1 and α2) and β-chain. The molecular weight of β-chain was heavier than α-chain (4).

Collagen from snakehead fish scale consist of two α-chains (α1 and α2) with the molecular weight approximately 118.032 kDa and 112.201 kDa. Moreover, a small amount of β chain with molecular weight 137.404 kDa dimerized by components were obtained in this collagen due to hydrolysis by proteases. This pattern was similar to the collagen previously reported from the skin of *Priyacanthus tayenus* that had molecular weights 118 KDa and 111 KDa (29). The diversity of collagen types is also caused by differences in gene expression in protein biosynthesis. In addition, posttranslational modification of collagen also has a significant effect on collagen diversity and its groupings, such as collagen type I (found in skin, tendon, and bone tissue), type II (found in cartilage), and type III (found in skin and vasculature) (4).

The fibrils formation profile shown in Fig. 5 from SEM visualization presented the collagen fibril morphology. The morphology showed random coil structure and obvious fibril networks with rough membranous structure for the collagen membrane. The result suggested that this collagen might undergo partial cleavage in the telopeptide region by protease treatment (31). The formation of fibril on collagen is contains hydroxylysine and subsequent O-glycosidic bonds which is modulators of the fibrilllogenesis process (5). The collagen consists of the matrix at the later stage that take place as microfilaments, fibrils, and mature collagen filament (4). This formation was influenced by the isolation method. Collagen was extracted in an acidic solution containing pepsin in an isolation process which only attacks the non-triple helix domain of the original collagen. Therefore, it has a small molecular weight without a triple-helix structure. Collagen monomer bonds in the form of fibrils make collagen hydrophobic and have electrostatic interactions (30).

This research showed collagen from *Channa striata* scales can be isolated enzymatically using a protease produced by *B. subtilis* which can be characterized as type 1 collagen. Thus, collagen from snakehead fish scales can be an alternative source of collagen for further applications in the food and nutraceutical industries.

Ethical Statement

None to declare.

Conflict of interest

The authors declares that there is no conflict of interest regarding the publication of this paper.

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Authors' contributions

Concept: S.N., Design: S.N., N.W., Data Collection or Processing: S.N., Analysis or Interpretation: S.N., N.W., Literature Search: S.N., Writing: S.N., N.W.

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