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Characteristics of Collagen-based Milkfish Bone Waste Extracted with Bromelain with Cofactor Ca^{2+}

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ABSTRACT

Indonesian imports of gelatin and collagen raw materials totaled 4808 tons. Gresik is one of the producers of milkfish in East Java, with 87116 tons expected in 2022. Even though it contains type 1 collagen, milkfish bone waste that is not used at the center for making milkfish brains is an environmental hazard. Given this possibility, this study aimed to determine the qualitative characteristics of collagen extract from milkfish bone debris (*Chanos chanos*) with and without the addition of Ca^{2+} of varying quantities. The extraction method employs the enzyme bromelain 2% and cofactor metal ion Ca^{2+} (0.5%; 1.0%; 1.5%), which has previously been pre-treated in the form of defatting and deproteination, before determining physical and chemical properties. The results showed that using bromelain enzyme with Ca^{2+} boosted collagen yield. Adding 0.5% Ca^{2+} resulted in the highest yield, 7.58±1.88%. All collagen produced contains functional groups recognized in FTIR as type 1 collagen constituents (presence of amide A, amide B, amide I-III). The melting point of the collagen generated is between 144 and 157 °C. The SEM profile of collagen was porous sheets in all treatments. Except for the ash content, the chemical properties of collagen generated by adding the Ca^{2+} (pH 7.49-8.09; water content 7.75-8.15; ash content 6.56-7.78) fulfill SNI and BSP standards. Meanwhile, collagen synthesized without the inclusion of cofactors only meets the water content standards (pH 7.49-8.09; water content 7.75-8.15; ash content 6.56-7.78). The demineralization stage is required to produce milkfish bone debris before extraction to achieve these requirements.

***Keywords*:** Bromelain; Cofactor; Ca^{2+}; Bone; Milkfish; Collagen

INTRODUCTION

In Indonesia, the development of the cosmetics business has not been balanced with the production of necessary raw materials. According to BPS, Indonesian imports of gelatin and collagen raw materials reached 4,808 tons in 2019 and continue to rise year after year.[1] To overcome this problem, studying Indonesia's natural resources is vital, aiming for health independence research related to discovering pharmaceutical raw materials. Gresik City is the leading producer of milkfish in East Java, with 87,116 tons expected in 2022.[2] Milkfish brains are thus one of the most popular typical Gresik souvenirs in Gresik. These brains are created by separating the fish meat from the bones, with the bone debris discarded. In reality, milkfish collagen is classed as type 1, which has high antioxidant activity.[3, 4, 5]

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Collagen is a fibrous protein molecule in tissue connectives and components that comprise the primary structural elements and components of body parts such as teeth, muscles, bones, and nails. Collagen is a water-soluble protein composed of three polypeptide chains in a triple helix conformation. Collagen is easily absorbed in the body, non-toxic, has a high water affinity, is biocompatible, biodegradable, reasonably stable, easy to produce, and soluble, and its use in industry is increasing rapidly. Collagen has antioxidant properties.[6] According to Silva *et al.* (2013), collagen usage can be employed in the cosmetic sector, including early anti-aging treatments, skin care products, and make-up in liquid (lotion), gel, or powder. [7]

Physical, chemical, and enzymatic procedures can extract collagen from milkfish bones.[8] The use of enzymes in collagen extraction has proven to be more effective in providing higher yields, particularly the protease group.[9,10] Protease enzymes are a class of enzymes that degrade proteins and lipids. This enzyme family is present in various plants, animals, microbes, and people. Bromelain enzyme is one of the protease groups derived from the pineapple plant (*Ananas comosus*). Bromelain converts peptide bonds in protein content into amino acids. It possesses features comparable to proteolytic enzymes, which can hydrolyze other proteins, such as renin, papain, and fisin. In the protein breakdown process, this enzyme has the advantage of being resistant to a wide pH range of 3-7 and temperatures ranging from 20 to 50 ^{o}C. [11] The use of enzymes in collagen extraction can boost the yield. According to Pamungkas *et al.* (2018), collagen yield is higher when extracted using the enzyme pepsin than when extracted with an acid solution. Extraction of collagen using the enzyme pepsin yielded 1.94%, while extraction of collagen with acid solution yielded 0.94%.[12]

Cofactors are one component that can boost enzyme activation energy. Some cofactors can stabilize the secondary structure of the bromelain enzyme, increasing its enzymatic activity.[13] An inorganic cofactor, such as the metal ion Ca^{2+}, is one form of cofactor that can be employed. This cofactor, known as an activator, is one of the cofactors frequently used to improve enzyme activity. [14]

The novelty of this research compared to previous studies was an additional cofactor in the bromelain enzyme in extracting collagen from waste milkfish bones. In this research, using Ca^{2+} as cofactor with a concentration of 0.5%; 1%; and 1.5%, so that the yield ratio was known collagen produced without/with the use of cofactors. Apart from that, most of them previous research had used specially purchased milkfish bones instead comes from waste. By utilizing this waste, it could be able to support efforts the government promotes zero waste but is still beneficial for health, in particular in the pharmaceutical sector (drugs and cosmetics). Based on this background, this basic research aims to obtain the quality characteristics of collagen extract from milkfish (*Chanos chanos*) bone waste with the addition of Ca^{2+} metal ion cofactors of various concentrations and without the cofactor in the bromelain enzyme.

METHODS

Instrument

Instrument used in this research include, among others, centrifuge (SC8 Boeco Germany), incubator (DSI-300D-China), oven (DSO-300D-China), analytical balance (Osuka type FA2004E), magnetic stirrer and stirrer (IKA C -MAG HS7), Meanwhile, for the analysis of collagen characteristics, the Melting Point Apparatus Fisher Scientific (Germany) was used. PERKIN ELMER Spectrum One Fourier-Transform Infrared JEOL RESONANCE 400 MH (Waltham, USA), FEI Inspect S50 (Oregon, USA).

Material

The materials used include milkfish bone waste from the Mak Cah and Bu Muzanah souvenir center in Gresik City; East Java province; Indonesia, sodium bicarbonate pa (Sigma Aldrich; St. Louis,

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MO), NaOH pa (Merck; Darmstadt, Germany), $CaCl_2$ pa (Merck ; Darmstadt, Germany), bromelain enzyme (Rainwood, Shanxi, China. Meanwhile, for analysis of collagen characteristics, KBr pa (Merck; Darmstadt, Germany) was used.

Procedure

Milkfish bone preparation

The milkfish bone waste obtained was prepared, which included *defatting* and deproteination. Defatting is done to remove fat from the spines by mixing the bones which have been reduced in size with 1.25 g sodium bicarbonate in 1000 ml and 200 g ice cubes. Next, *deproteination* was carried out to remove other proteins in the bones which was carried out by mixing with NaOH (0.1; 0.5; and 1) M, where each concentration was replaced every 1 hour. After pre-extraction, the extraction process continues. [15]

Extraction method

The extraction stage of this research was that 80 g of milkfish bone powder was weighed 12 times, then put into each 250 ml beaker. 160 ml of bromelain enzyme solution was added to each 250 ml beaker glass and Ca^{2+} metal ions as in Table 1. Then, each beaker glass was covered with aluminum foil and placed in the incubator for 2.5 hours. Next, filter it with flannel cloth to obtain a filtrate. Next, it was centrifuged using a centrifuge and the supernatant was obtained which was evaporated using an oven at T = 50 ^{o}C. [16] After that, the dry collagen formed was constantly weighed.

Table 1. Addition of Ca^{2+} metal ions

[Metal Ion Ca^{2+}] (%)	Label
0.5	4, 5 and 6
1	7, 8 and 9
1.5	10, 11 and 12

Physical and chemical characteristics testing

The resulting collagen was tested for its physical and chemical characteristics. Physical characteristics include melting point by observing the end point of collagen melting from four treatments, FTIR identification [17] was carried out on two treatments, namely the highest amount of collagen extract (bromelain with Ca^{2+} metal ions) and bromelain without cofactors. Meanwhile, SEM characterization was tested at the Material Characterization Division of the Sepuluh Nopember Institute of Technology (ITS). Chemical characteristics including pH, ash content [18] and water content [19] tests were carried out on all cofactor treatments (0.5%, 1%, 1.5%) and without cofactors.

RESULTS AND DISCUSSION

Collagen yield uses the enzyme bromelain with and without cofactors

Collagen extraction from milkfish bones in this study was carried out enzymatically, namely using the enzyme bromelain. The enzymatic method is considered to be able to speed up extraction time and increase the amount of collagen yield produced. The yield of collagen extracted using bromelain was 5.46 ± 1.97. The yield of bromelain soluble collagen is greater than the extraction research with acetic acid with a maximum of 1.84%.[20] with the bromelain enzyme alone at 6%,[21] and compared to pepsin and papain which was less than 1%. [22] Differences in collagen extraction yield results can be influenced by the characteristics of the raw material, enzyme activity values, pH, extraction time and extraction temperature. The process of dissolving collagen by the enzyme bromelain occurs due to the breakdown of *cross-linking* molecules in polypeptides without damaging the integrity of the collagen triple helix.

In this research, a cofactor was added in the form of the metal ion Ca^{2+} , which is a type of inorganic cofactor. The yield of bromelain soluble collagen with the addition of Ca^{2+} metal ions obtained the results as shown in Table 2.

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Table 2. Collagen yield with variations in Ca^{2+} cofactor concentration

[Metal Ion Ca^{2+}] (%)	Yield Results (%)
0.5	7.58 ± 1.88
1	6.21 ± 1.53
1.5	6.72 ± 1.19

The yield of bromelain soluble collagen with the addition of the Ca^{2+} cofactor resulted in an increase in yield when compared to without the cofactor, namely 5.46 ± 1.97. The metal ion Ca^{2+} works by attaching to the active site of bromelain, so that it can complement and modify the structure of the bromelain enzyme. The attachment of this cofactor can increase the performance of bromelain to open collagen fibers and dissolve collagen without damaging the integrity of the collagen triple helix. The addition of the Ca^{2+} cofactor was 0.5% greater than the other two concentrations, namely 1% and 1.5%. The concentration of cofactors that interact with bromelain can affect the yield of collagen, where the greater the concentration of metal ions added, the greater the activity of bromelain in dissolving collagen. This is because the enzyme is in a saturated state when the concentration of added metal ions increases.

Physical characteristics of the collagen produced

Collagen melting point

Collagen is a polymer of protein that can denature into monomers in the presence of heat. The melting point produced using the Fischer Melting Temp apparatus is the peak melting temperature of the collagen triple helix.

The results in Table 3 show that there is a significant difference in the melting point of collagen from milkfish bone waste between the treatment groups using the one-way ANOVA test (sig < 0.05). The melting point results obtained were lower than the results for *lizard* fish bone collagen (*Saurida tumbil* Boch) 180.42-212.11 ºC with acid variations. [23] The higher the melting point temperature of the triple helix, the more thermally stable the collagen.

Table 3. Collagen melting point test results resulting from bromelain extraction with several cofactors

Treatment	Cofactor concentration (%)	Melting Point (º C)
Ca^{2+} cofactor	0.50%	144 ± 1.53
	1.00%	148±1.57
	1.50%	157±1.54
No cofactors		152±1.58

The increase in collagen stability is due to the presence of intramolecular hydrogen bonds in the triple helix structure which remains stable during extraction treatment. [24] However, in this study it had a lower melting point. This can be caused by the lack of the amino acids proline and hydroxyproline in milkfish bones where these two amino acids will form intramolecular hydrogen bonds and help maintain thermal stability in the collagen triple helix chain, resulting in a decrease in the melting point. [25]

Identification of collagen marker functional groups using FTIR

Collagen can be identified using FTIR through analysis of its functional groups. Fish bones have specific types of collagen functional groups, namely Amide A, Amide B, Amida I, Amida II, and Amida III.[26] The results of the analysis of collagen functional groups resulting from bromelain extraction with 0.5% Ca cofactor metal ions (figure 2) and without cofactors can be seen in table 4. From these results, in the amide A functional group, both collagens have absorption peaks which fall within the absorption region according to between 3300-3500 cm^{-1} , namely the addition of a cofactor (3444.58 $cm^{-1)}$ with no cofactor (3456.74 cm^{-1}).[27] The presence of amide A groups, the stretching vibration characteristics of -NH, is influenced by the presence of hydroxy groups in water molecules that participate in the collagen structure.[28] However, when the NH group is involved in hydrogen bonding in the peptide chain, its position can shift to a lower frequency. This causes differences in

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the shift in collagen absorption with cofactors compared to without cofactors.

Table 4. Identification of functional groups by FTIR of collagen extract from milkfish bone waste

Amides	Absorption area (cm^{-1})	Absorption peak		Information
		No Cofactors	+$CaCl_2$ cofactor 0.5%	
Amida A	3300-3500	3456.74	3444.58	NH stretching vibrations
Amide B	2915-2935	2925.77	2925.97	Asymmetrical stretching of CH2
Amida I	1620-1800	1745.05	1744.73	C=O stretching vibrations
Amide II	1590-1650	1591.92	1591.67	NH bending, CH stretching
Amide III	1229-1301	1276.10	1276.75	CH stretching, NH bending

Amide B functional group with asymmetrical CH2 stretching characteristics in collagen produced with the addition of Ca^{2+} cofactor (2925.97 cm^{-1}) with no cofactor (2925.77 cm^{-1}) is by the range of previous research between 2915-2935 cm^{-1}.

Furthermore, there is a lower shift in the stretching vibration characteristics of the C=O group of Amide I groups between collagen produced with the addition of the Ca^{2+} cofactor (1744.73 cm^{-1}) and without the cofactor (1745.05 cm^{-1}) following the absorption area research. [29]

The C=O group which is involved in hydrogen bonds can also cause a lower absorption shift such as the Amide A group. The Amide II group does not experience too large a shift with NH bending and CH stretching characteristics

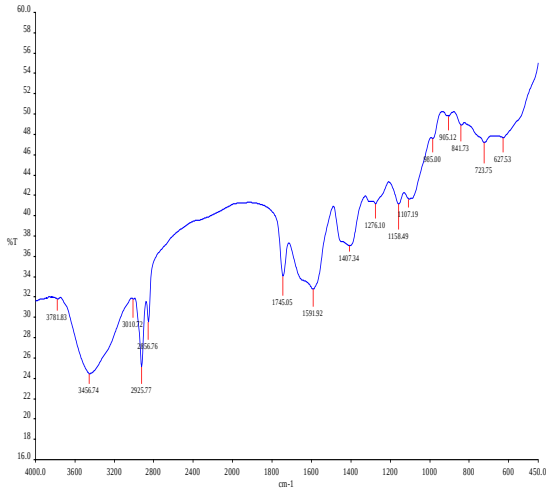

Figure 1. FTIR spectrum of collagen resulting from 2% bromelain enzyme extraction without cofactor

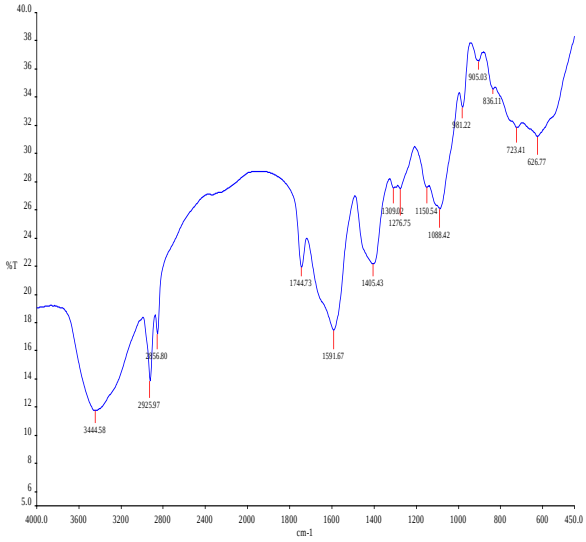

Figure 2. FTIR spectra of collagen resulting from 2% bromelain enzyme extraction with cofactors $CaCl_2$ 0.5%

between the results of the addition of the Ca^{2+} cofactor (1591.67 cm^{-1}) without cofactor (1591.92 cm^{-1}) and still within the absorption range of 1590-1650 cm^{-1}. The amide III group with the characteristics of CH stretching and NH Bending also does not have too big a shift towards the collagen produced by adding the Ca^{2+} cofactor (1276.75 cm^{-1}) with no cofactor (1276.10 cm^{-1}) following the research range previous. [30]

Morphology using SEM

Morphological analysis of collagen produced with the addition of Ca^{2+} cofactor and without cofactor at 500 x

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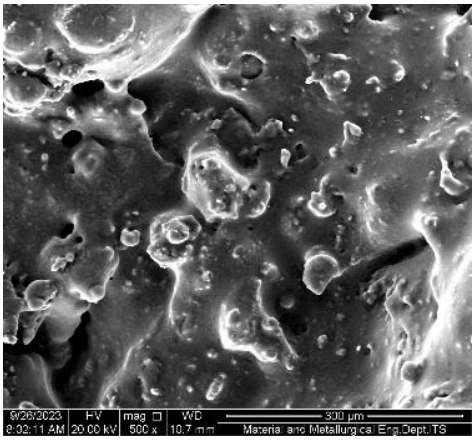
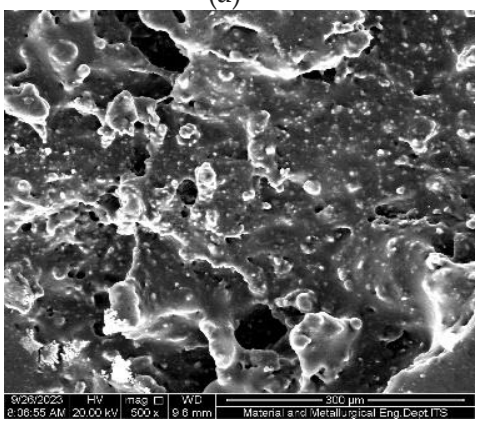
magnification can be seen in Figure 3. Based on these results, both have the same shape, namely in the form of sheets with a porous surface. However, the pores between collagen extracted from the addition of the Ca^{2+} cofactor have more pores than without the cofactor. These pores are produced due to the presence of spaces between collagen fibers .[31] Where the space between the fibers can increase the solubility of collagen. The results of these morphological observations are useful for knowing the characteristics of collagen which can later be applied to formulate cosmetic preparations.



(a)



(b)

Figure 3. SEM results of the physical characteristics of collagen in the form of sheets with a magnification of 500 x produced by extraction with (a) bromelain and (b) bromelain with Ca^{2+} cofactor

Chemical Characteristics of Collagen

The chemical characteristics of collagen are required for the raw material to meet the Indonesian National Standards (SNI). The components tested on extracted collagen include pH, water content and ash content. The results of chemical testing of collagen produced with the addition of Ca^{2+} cofactor and without cofactor are as shown in table 5.

Table 5. Characteristics of pH, water content and ash content of collagen

Treatment	Cofactor concen-tration	pH	Water content	Ash content
+$CaCl_2$ cofactor 0.5%	0.50%	8.09 ± 0.00	8.15 ± 0.02	7.27 ± 0.01
	1.00%	7.91 ± 0.01	7.34 ± 0.05	6.56 ± 0.01
	1.50%	7.49 ± 0.00	7.75 ± 0.04	7.78 ± 0.02
No cofactors	-	9.02 ± 0.00	6.52 ± 0.03	8.71 ± 0.01

The results of the pH characteristics of this study ranged from 7.49-9.02 in the four treatments. The BSN standard (2014) for dry collagen is 6.5-8. Where only collagen produced from bromelain extraction without cofactors does not meet these requirements because it reaches 9.02. [31] The pH value of this study is also higher than of 5.34 with the extraction method using acid. This is likely due to the large amount of minerals still contained in collagen. This statement is supported by the results of ash content in research that does not meet SNI standards (<1%) in the range of 6.56-8.71. This result is due to the large mineral content in collagen and can be minimized through demineralization at the initial stage of extraction. The dry collagen produced by this research still contains minerals because no mineral separation was carried out before deproteinization. If minerals are not separated during preparation, they will still be detected in the collagen so that when tested the ash content will be calculated as the sum of the ash content. [32]

The characteristics of water content in all treatments were between 6.52-8.15, this result still met the maximum SNI standard of ≤ 12%. In the research, the water content between the collagen treatments extracted without the cofactor was lower than with the addition of the Ca^{2+} cofactor. The cause of the low water content of collagen from bones is that the collagen structure in bones

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is stronger and denser so the use of acid during extraction cannot hydrolyze collagen. [31] This is reinforced by the SEM results on the physical characteristics where collagen resulting from bromelain extraction with the addition of the Ca^{2+} cofactor has more pores, so water will easily be trapped in the structure.

CONCLUSION

Enzymatic collagen extraction using the enzyme bromelain with the addition of Ca^{2+} cofactor (0.5; 1; 1.5%) increased collagen yield. The addition of 0.5% Ca^{2+} cofactor produced the largest yield, namely 7.58 ± 1.88%. Meanwhile, the Characterization of physical and chemical collagen fulfilled SNI and BSP standards, except for ash content. To achieve these needs, future studies should include a demineralization stage in the production of milkfish bone debris before extraction.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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