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OPTIMIZATION OF PAPAIN SOLUBLE COLLAGEN EXTRACTION FROM MILKFISH SCALES (CHANOS CHANOS FORSKAL)

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ABSTRACT
This study aims to determine the papain soluble collagen from the milkfish scale based on the yield and antioxidant activity due to treatment of extraction time and papain concentration. Papain soluble collagen (PSC) was isolated from the scales of milkfish (*Chanos chanos*) caught in Sidoarjo, Indonesia, with a total production of 828,372.62 tons in 2018. Independent variables of extraction time were (X_1, hour) and papain concentration (X_2, U/mg). Dependent variables were yield (Y_1, %) and antioxidant activity (Y_2, mg/L).This research used Response Surface Methodology. The result showed that the extraction time and papain concentration from 24 hours to 82 hours with papain concentration 3000 U/mg to 7000 U/mg resulted in yield 2.25% to 6.4% and antioxidant activity of 199 mg/L to 418 mg/L. The optimal condition of extraction PSC was 56.45 hours with papain concentration 5950 U/mg resulting in a yield of 6, 0%±0,012 and antioxidant activity of 258±0, 98 mg/L.

KEY WORDS
Collagen, papain, fish scales.

The fishing industry, both from fishing activities and processing businesses, causes significant by-products and waste that has not been utilized until now. The production of fresh boneless milkfish results in solid waste by 25% in scales, offal, bones, and spines. Solid waste scales produce 4.7% of the initial weight of raw materials. Scales waste is still limited as fish and animal feed material thrown into the environment can cause environmental pollution (Suseno, 2011).

One alternative to the use of milkfish scales is to make them a safe source of collagen. Collagen is part of the extracellular matrix protein that plays an essential role in maintaining the structure of various tissues, in the form of the main components of various connective tissues such as skin, bones, tendons, blood vessels, basal membranes, cartilage, and teeth that account for about 25- 30% of the total protein content (Astiana,et al., 2016; Pati et al.,2010). Fish skin and fish scales have type-1 collagen, similar to human skin (Hsiung Pan *et al.,* 2010). Collagen from marine fish can be commercially produced from the skin and scales of fish. Type I collagen can be widely applied in various food, biomedicine, pharmaceutical, and cosmetic industries (Hoyer et al., 2014).

Several methods of making collagen from the skin and fish scales can be done through the conventional extraction process using solvents or enzymatically (Hartati and Kurniasari, 2010). Enzymes are biocatalysts that have been widely used in the industrial field because enzymes can speed up a reaction without reacting. In industries that use enzymes, 59% of the enzymes used are proteases, one of which is papain (Soda et al., 2013).

Papain's ability to break down protein molecules, making it a product that is very beneficial for human life both in household and industry, such as in the process of meat, the manufacture of protein concentrates, the manufacture of curds, skin softeners in the industry from papaya sap, both in fruit, stems and leaves. Young papaya stems, leaves, and fruit contain white sap. More than 50 amino acids in papaya sap, including aspartate acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, isoleucine, leucine, tyrosine, phenylalanine, histidine, lysine, arginine, tryptophan, and cysteine. In addition, sap also contains a protein-breaking enzyme or proteolytic enzyme called papain (Indriani et al., 2008).

Some research on collagen extraction from fish processing by-products that have been done includes acid-soluble collagen and pepsin from dried scales (*Channa striatus*) dry (Pamungkas et al., 2018). (Li et al*,* 2013), also reported acidic soluble collagen and pepsin from the skin and bones of Spanish mackerel, skin, and bubble swimming grass carp, and on Seabass scales (Wu et al., 2015; Chuaychan et al., 2015). Nurilmala et al. (2019) using the enzyme papain for collagen extraction of tuna skin. Several acid-soluble collagen studies have also been conducted from yellow-tailed fish skin (Astiana et al., 2016), African catfish skin, salmon, Baltic cod (Tylingo et al. 2016), and patin fish skin (Devi et al*.,* 2017), as well as milkfish scales, have also been reported by (Wahyu and Widjanarko, 2018).

The use of milkfish as a processed product leaves waste, especially fish scales that reach 30-40% of raw materials and can cause environmental pollution if the disposal system is incorrect. Optimal utilization of fish processing by-products is primarily as a raw material for collagen, increasing the added value of by-products and reducing the environmental pollution. Milkfish scales can be used as an alternative source of raw materials for collagen extraction. However, there has not been much research on collagen extraction from milkfish scales using enzymatic extraction methods. This research was conducted to optimize Papain Soluble Collagen Extraction on Milkfish Fish Scales *(Chanos chanos*).

MATERIALS AND METHODS OF RESEARCH

The scales were from milkfish by-product of boneless milkfish production by Teaching Factory Modern Processing Marine Polytechnic and Fisheries Sidoarjo. Scales from the production of the boneless milkfish were then cleaning and washing. Handling fish scales was done by paying attention to the temperature that was the temperature of ≤ 4°C. The scales of the milkfish fish were then stored in cold storage with a temperature of -18°C ±2°C. Other materials used in the study were sodium hydroxide (NaOH), papain enzyme (Merck), acetic acid (CH_3COOH), sodium chloride (NaCl), trichloroacetic acid (Merck), aquades, EDTA2NA, 2,2-diphenyl-1-pikrilhydrazil (DPPH) test, Ascorbic acid and ethanol.

The process of extracting collagen soluble papain first scales was done pretreatment based on (Wahyu, 2018) method with NaOH for 8 hours with a ratio of 1: 8 (w / v) and carried out the washing process until the pH was neutral. The following process was to be demineralized using a solution of 0.5 M EDTA-2Na (pH 7.5) for 24 hours with a ratio of 1: 8 (w / v), then carried out the washing process (Wahyu and Widjanarko, 2018). Fish scales were then extracted with the enzyme papain 3000 U / mg up to 7000 U / mg and added a solution of CH_3COOH 0.6 M with a time of 24 hours to 72 hours. Next, the sample was filtered and taken supernatant. Precipitation was carried out with NaCl until the final concentration of the solution reached 0.9 mol /L until collagen expression (salting out) was obtained. The compressive was concentrated at 2000 rpm at 4°C for 20 minutes. The centrifuge results were dialysis with dialysis bag 12 kda in acetic acid solution 0.5 M and 0.1 M. The extraction process was carried out with a temperature of 10 ° C. Then, collagen was lyophilized using freeze-drying (Wahyu and Widjanarko, 2018).

Optimization was done with Response Surface Methodology (RSM) and Central Composite Designs (CCD) models. The variables in this study were the free variable papain enzyme (X_1) with a concentration of 3000 to 7000 U/mg, the time (X_2) used was 24 hours to 72 hours. Bound variables were yield (Y_1) and antioxidant activity (Y_2).

Table 1 – Research Design

Variable	Code of Treatment				
	-α	-1	0	1	+α
Concentration of papain (U/mg)	2000	3000	5000	7000	8000
Time of extraction (hour)	14	24	48	72	81

The determination of the minimum point (code -1), the maximum point (code +1), and the midpoint (code 0) were obtained based on previous research and preliminary research. The Central Composite Design (CCD) was used to determine the optimization conditions of

the papain soluble collagen extraction process from the scales of the milkfish (*Chanos chanos*). The experimental design used at this stage is in Table 1.

Collagen yield calculation was obtained by comparing the dry weight of collagen scales of milkfish with the raw material weight of milkfish scales before being isolated. The yield could be obtained by formula [13].

$$\text{Collagen yield (\%)} = \frac{\text{Dry weight of collagen}}{\text{Raw material weight}} \times 100\%$$

The antioxidant activity test was applied following method [3]. This study adopted free radical antidote activity using the 2,2-diphenyl-1-pikrilhydrazil (DPPH) test. Ascorbic acid was used as a positive control and ethanol as a negative control. Absorption was measured at 514 nm.

RESULTS AND DISCUSSION

This treatment determined the concentration of enzymes and the right time to obtain collagen with the highest yield and antioxidant activity. Analysis of 13 treatments obtained results such as Table 2.

Table 2 – Results of Long Analysis of Extraction Time with Concentration of Papain Enzymes

No	Level Parameters		Extraction Parameters		Response	
	X_1	X_2	Extraction time (hours)	Concentration of Papain Enzymes (U/mg)	Yield (%)	Antioxidant Activity (mg/L)
1	-1	-1	24	3000	2.33	434
2	1	-1	72	3000	2.56	214
3	-1	1	24	7000	4.81	249
4	1	1	72	7000	6.20	297
5	-1,414	0	14	5000	2.25	418
6	1,414	0	82	5000	4.13	199
7	0	-1,414	48	2171	3.88	292
8	0	1,414	48	7828	6.40	255
9	0	0	48	5000	6.13	270
10	0	0	48	5000	5.90	260
11	0	0	48	5000	6.05	258
12	0	0	48	5000	5.60	231
13	0	0	48	5000	5.63	238

Table 3 – Response Surface Model for Yield and Antioxidant Activity

Response	Model	Significant (p<0.05)	Lack of Fit (p<0.05)	R^2
Yield	Quadratic	<0.0001	0.070	0.96
Antioxidant Activity	Quadratic	0.00004	0.133	0.94

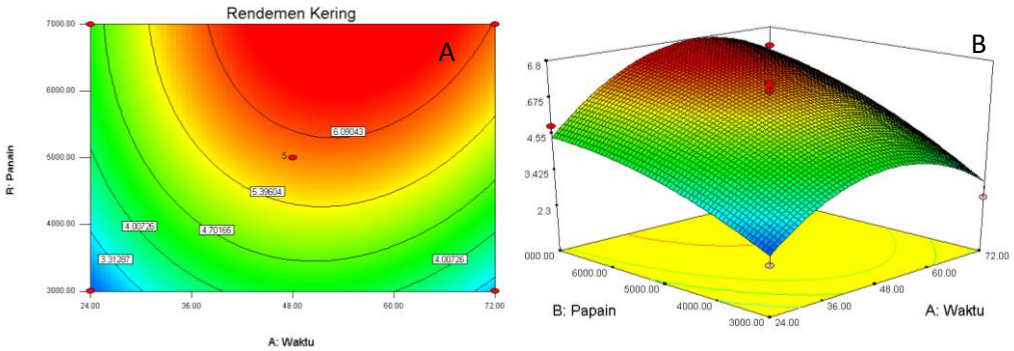

Figure 1 – Graph of Yield Response to Length of Extraction Time and Concentration of Papain: A = Contour Plot; B = 3D Curve

Table 4 – Analysis of Variance (ANOVA) from RSM yield and antioxidant activity

Response	Source	Sum of Squares	Df	Mean Square	F Value	P-Value, Prob>F
Yield	Model	28.08	5	5.62	33.63	<0.0001
	X_1-Time	2.05	1	2.05	12.28	0.0099
	X_2-Papain	11.71	1	11.71	70.14	<0.0001
	X_1X_2	0.32	1	0.32	1.95	0.2057
	X_1^2	13.30	1	13.30	79.67	<0.0001
	X_2^2	1.12	1	1.12	6.70	0.0361
	Residual	1.17	1	0.17		
	Lack of Fit	0.94	1	0.31	5.34	0.0696
	Pure Error	0.23	1	0.058		
	Cor Total	29.25	12			
Activities of Antioxidant	Model	57017.26	5	11403.45	21.12	0.00004
	X_1-Time	28246.38	1	28246.38	52.31	0.0002
	X_2- Papain	2975.97	1	2975.97	5.51	0.0513
	X_1X_2	17956.00	1	17956.00	33.25	0.0007
	X_1^2	6441.79	1	6441.79	11.93	0.0106
	X_2^2	1166.69	1	1166.69	2.16	0.1850
	Residual	3779.66	1	539.95		
	Lack of Fit	3779.66	1	906.82	3.42	0.1327
	Pure Error	1059.20	1	264.80		
	Cor Total	60796.92	12			

The yield of papain-soluble collagen based on Table 2 reaches 6.4%. Analysis of model selection shows that the Quadratic model is a recommended model for the analysis of the yield response (Y_1) to the extraction period factor (X_1) and the concentration of the enzyme papain (X_2). R^2 to the yield response is 0.96 and lack of fit 0.070 as in Table 3. The quadratic model exerts a significant effect on the yield, as in Table 4. The program provides an equation to the yield response in the form of actual variables:

$$Y = -4.89830 + 1.32137E\text{-}003\ X_1 + 1.32137E\text{-}003X_2 + 5.93750E\text{-}006\ X_1\ X_2 – 2.44746E\text{-}003\ X_1^2 – 1.00138E\text{-}007\ X_2^2$$

The effect of the length of extraction time and concentration of the enzyme papain can be seen on the yield response graph as in Figure 1.

Extraction time between 24 hours to 82 hours and the concentration of papain enzymes used ranging from 3000 u / mg to 7000 U / mg resulted in a yield of 2.25 to 6.4%. The process of extracting collagen with increasing time and high concentrations causes collagen production to be higher. First, the enzyme will abstract water-soluble proteins, then proteins that are not soluble in water. Initially, the extraction process dashes, but the longer the extraction time causes the speed of hydrolysis to decrease to the stationary phase.Yields using papain enzymes in certain phases will stagnate because the availability of substrates that bind to enzymes will decrease with the extended extraction time (Bisswanger, 2014). After the substrate is bound, the longer the extraction of the reaction will decrease because the enzyme binds no substrate (Vitolo, 2020).

In acidic soluble collagen on the scales of milkfish is optimal at a concentration of CH_3COOH 0.66 M for 61.30 hours with a yield of 0.7% (Wahyu and Widjanarko, 2018). Collagen yield produced from tuna skin using the enzyme papain as much as 7000 U / mg produces a yield of 22.79% (Nurilmala et al., 2019). The concentration of enzymes will not damage collagen proteins but will produce high purity, and collagen will have physical and chemical stability. The combination of acid treatment and papain enzyme will give more results in terms of yield and quality % (Nurilmala et al., 2019).

From Table 2, we know that with the concentration of papain enzymes ranging from 3000 to 5000 U / mg and the length of extraction time of 14 hours to 82 hours obtained antioxidants 199 mg / L to 418 mg / L.

Analysis of the selection of models shows that the Quadratic model is a recommended model for the analysis of the response of antioxidant activity (Y_2) to the extraction time factor (X_1) and the concentration of the enzyme papain (X_2), R^2 0.94, and lack of fit 0.133 in the antioxidant activity response as in Table 3. The quadratic model exerts a significant influence

on the antioxidant activity, as in Table 4. The program provides an equation to the response of antioxidant activity in the form of actual variables: $Y = -958{,}91015 - 0{,}10899\ X_1 + 1{,}39583E\text{-}003\ X_1X_2 + 0{,}038565\ X_1^2 + 3{,}23474E\text{-}007\ X_2^2$

The influence of the length of extraction time and concentration of the enzyme papain can be seen on the graph of antioxidant activity response as in Figure 2.

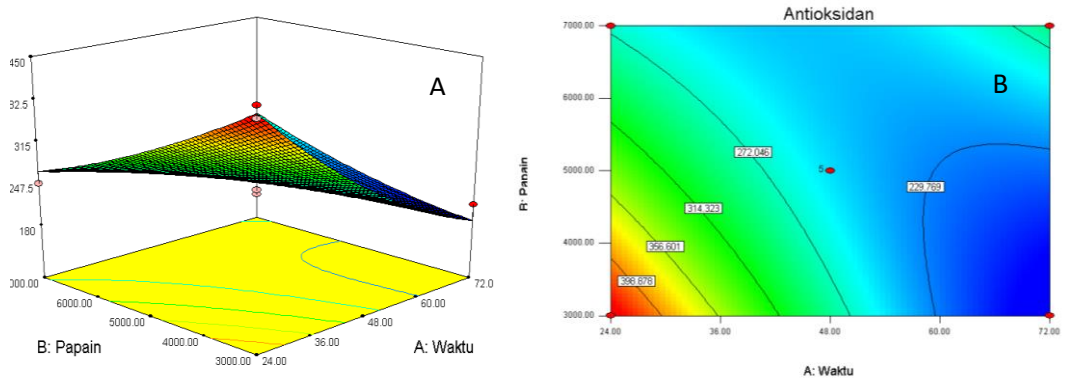

Figure 2 – Graph of Antioxidant Activity Response to Length of Extraction Time and Concentration of Papain: A = Contour Plot; B = 3D Curve

From Figure 2, we can know that the extraction time between 24 hours to 82 hours and the concentration of papain enzymes used ranging from 3000 u / mg to 7000 U / mg produce antioxidants of 199 to 418 mg / L. Differences in the concentration treatment of the enzyme papain resulted in different percent inhibition of DPPH free radicals. It indicates a relationship between antioxidant activity and the percentage of enzymes used in the hydrolysis process. Adding to the concentration of enzymes, the value of this percentage (inhibitory percent) increases to the enzyme concentration of 4%.

The increase in enzyme concentration will align with the number of peptides and free amino acids. When the use of enzymes increases, the number of peptides and free amino acids produced increases, so the value of the resulting antioxidants will also increase (Baehaki et al., 2015). Stated that the percentage of inhibition (percent inhibition) of free radical activity would increase along with the increase in extract concentration (Hanani et al., 2005). Bordbar (2013) also added that the size of peptide and its solubility, amino acid composition, strands, and the abundance of free amino acids are the keys that determine the capture capacity of DPPH radicals.

Antioxidants in the skin collagen of tuna fish produce 283 mg / L with a concentration of papain enzyme of 7000 U / mg. Collagen hydrolyzate shows that antioxidants are three times higher than collagen alone. The use of enzymes does not damage collagen proteins, produces high purity, and has good physical and chemical stability (Nurilmala et al., 2019).

The criteria of the response are the yield as much as possible. Determination of optimum point based on analysis of Design Experts as in Table 5.

Table 5 – Optimum Point Solutions from Design Experts

Solution	Time (hours)	Concentration (U/mg)	Yield (%)	Antioxidant (mg/L)	Desirability	Statement
1	56.45	5950	6.4	239	0,911	Selected

Table 6 – Verification of Optimum Point

n/n	Extraction time (H)	Papain (U/mg)	Respond of	
			Yield (%)	Antioxidant (mg/L)
Prediction	56.45	5950	6.4	239
Results	56.45	5950	6,0±0,012	258±0,98
The difference in predictive response value with research results (%)			0,94	1,08

Based on Table 5, the Design Experts program provides a solution, namely an extraction time of 56.45 hours with a concentration of the enzyme papain 5950 U / mg, which is predicted to produce a yield of 6.4% and antioxidants 239 mg / L.

Verification is done to determine conformity with the predictions of optimization of the Design Experts program. Table 6 data shows that the value of predictions with research results of 1% indicates that the prediction results are by the study's results or not different from actual.

CONCLUSION

The results of collagen extraction optimization from milkfish scales with extraction time between 24 hours to 82 hours and the concentration of papain enzymes used ranging from 3000 u / mg to 7000 U / mg resulted in a yield of 2.25 to 6.4% and antioxidants of 199 to 418 mg / L. Optimum extraction was obtained within 56.45 hours with a concentration of the enzyme papain of 5950 resulting in a yield of papain soluble collagen of 6.0% ±0.012 and antioxidants of 258±0.98 mg / L.

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