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Bioactive Peptide Activity of Indonesia Native Duck Feet Collagen Hydrolisate and its Potential as an Antioxidant Agent

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ABSTRACT

Collagen is the main structural protein found in animal bodies. Collagen can be extracted from various sources, including animal skin and bones. One potential raw material as a source of collagen is local duck feet. This study aims to determine the activity of bioactive peptides produced from the best collagen extraction results using bromelain enzyme followed by hydrolysis using Bacillus subtilis enzyme. The samples used were local duck feet with a harvest age of 1 to 2 years. This study consisted of two main stages, namely the extraction and isolation of collagen with soaking times of 12, 18, and 24 hours and different bromelain enzyme concentrations of 0%; 0.3%; 0.6%; 0.9%. The variables observed included: crude yield, protein concentration, Fourier Transform Infrared Spectroscopy (FTIR) functional group analysis, Differential Scanning Calorimetry (DSC) analysis, and Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) molecular weight analysis. The best results of phase I research were obtained from collagen extraction over a period of 24 hours with a Bromelin enzyme concentration of 0.9%, followed by hydrolysis using a 5% protease enzyme from Bacillus subtilis with an incubation time of 120 minutes. The characteristics of the collagen hydrolysate were tested using the ninhydrin test, followed by a % DH calculation. The antioxidant activity of collagen hydrolysate was tested using the DPPH free radical scavenging method. The results showed that collagen hydrolysate derived from local duck feet had bioactive peptide activity and potential as a strong antioxidant agent with an IC50 value of 98.07 µg/mL.

Keywords: Local duck feet, collagen, collagen hydrolysis, bioactive peptides, antioxidants

Introduction

Industrialization has had a major impact on increasing environmental pollution and the accumulation of free radicals in the air. High exposure to free radicals can attack normal cells and damage cell membranes and organelles, triggering an imbalance between the number of free radicals and the body's antioxidant defense system. This condition causes oxidative stress, which has the potential to reduce cell function and increase the risk of degenerative diseases. Sari et al. (2018) explain that excessive oxidation reactions in the body can be the initial trigger for various health disorders because highly reactive free radicals can damage cell structure and function.

Antioxidants are compounds that play a role in preventing or slowing down cell damage caused by free radicals through mechanisms such as electron donation, free radical scavenging, and inhibition of oxidative chain reactions. Pratiwi et al. (2023) state that antioxidant compounds are able to counteract the effects of free radicals and stabilize them by completing the electron deficiency in these radicals. Antioxidant sources are widely found in natural ingredients such as vitamin C, vitamin E, polyphenols, and bioactive peptides (Susanto et al., 2018). One naturally occurring antioxidant source that has been extensively studied is protein hydrolysate, which is known to have the ability to scavenge free radicals and suppress lipid oxidation processes.

Collagen is one of the main structural proteins in animal bodies that is widely used in various fields, especially food and pharmaceuticals, as a source of bioactive peptides. Afifah et al. (2023) mention that collagen can be extracted from animal skin, bones, tendons, and connective tissue. Local duck feet are one of the animal body parts with high

collagen content, making them a potential raw material for the production of bioactive peptides. Bioactive peptides produced from the hydrolysis of local duck feet collagen have various biological activities, including as antioxidant.

Previous studies have shown that protein hydrolysates contain bioactive peptides that play an important role in maintaining health and reducing the risk of disease (Hidayat et al., 2018). Samaranayaka and Li-Chan (2011) also reported that protein hydrolysates have antioxidant activity through the mechanisms of free radical scavenging, proton donation, and metal ion binding. Several studies on other commodities also reinforce this potential, including Puspawati et al. (2020), who successfully hydrolyzed chicken skin protein and obtained strong antioxidant activity with an IC_{50} value of 92.98 ppm. Wahyuni (2019) study on local goat skin collagen hydrolysate also showed high antioxidant activity with an IC_{50} value of 54 µg/mL.

The use of collagen from local discarded duck feet is still very limited. Scientific studies that optimize the collagen extraction process and evaluate its effectiveness in producing antioxidant bioactive peptides have also not been widely conducted. Therefore, research on hydrolyzed collagen from local duck feet is urgently needed to explore its potential as a natural antioxidant source with high added value for the livestock-based food industry and the health sector.

Based on this, this study was conducted to determine the bioactive peptide activity produced from the hydrolysis of local discarded duck feet collagen using *Bacillus subtilis* enzymes, as well as to evaluate the antioxidant activity potential of the peptides formed. The results of this study are expected to provide a scientific basis for the development of functional food products based on bioactive peptides from animal sources, particularly from local discarded duck feet waste that has not been optimally utilized.

Material and Methods

Analysis of raw materials and sample preparation

The first stage of the study began with an analysis of the chemical composition of discarded local duck feet to determine the ash, protein, and fat content using the AOAC (2005) standard method. Duck feet aged 144 weeks were obtained from farmers in Bantul. The duck feet were cleaned of nails and washed thoroughly, then ground. The collagen isolation procedure followed the method of Cheng et al. (2009) with several modifications. The samples first underwent a defatting process in 20% ethanol for 24 hours to remove fat. After that, they were soaked in 0.2 M NaOH for 24 hours to remove non-collagen proteins. The extraction stage was carried out by soaking the samples in a 0.5 M acetic acid solution with added bromelain enzyme at four different concentrations (0%; 0.3%; 0.6%; 0.9%) and three different soaking times (12, 18, and 24 hours) at a temperature of 4°C. The extract was then precipitated using 1.25 M NaCl. Next, a dialysis process was carried out for 72 hours and dried with a freeze dryer to produce dry collagen.

Caracterization of collagen

The second stage aims to determine the quality of collagen produced through several characterizations. Collagen yield is calculated from the ratio of dry collagen weight to fresh material weight. Protein concentration was analyzed using the Waddell method based on absorbance at wavelengths of 215 nm and 225 nm. FTIR spectrum analysis was performed to identify the main functional groups of collagen, including amide regions A, B, I, II, and III, using KBr pellets as the medium. The thermal stability of collagen was tested using Differential Scanning Calorimetry (DSC) in the temperature range of 20–445°C with a heating rate of 10°C/minute to observe endothermic peaks and denaturation points. Meanwhile, molecular weight analysis was performed using the SDS-PAGE method following the Laemmli (1971) procedure to identify the β, α1, and α2 protein bands characteristic of type I collagen. The research design at this stage used a 3×4 factorial pattern and the data were analyzed using ANOVA and a DMRT follow-up test if there were significant differences between treatments.

The results of the characterization at this stage were used to determine the best treatment to proceed to the hydrolysis stage.

Collagen hydrolysis and antioxidant activity test

After obtaining the best collagen from the previous stage, the third stage was carried out using a hydrolysis process based on the method of Zhang et al. (2018) with modifications. A total of 7 mg of collagen was dissolved in 10 mL of distilled water, then hydrolyzed by adding 5% (v/v) protease enzyme from Bacillus subtilis at a temperature of 50°C for 120 minutes. The reaction was stopped by heating at 90°C for 10 minutes to inactivate the enzyme, then the mixture was centrifuged at 8000 rpm for 15 minutes and the supernatant was taken as collagen hydrolysate. The free amino acid content was tested using ninhydrin reagent at 90°C with absorbance reading at 570 nm. The degree of hydrolysis (%DH) was determined using the 0.44 M TCA method, followed by measurement of the soluble protein concentration using the Bradford method. The antioxidant activity of the hydrolysate was tested using the DPPH method with a concentration variation of 1–200 µg/mL, then the absorbance was measured at 517 nm to calculate the percentage of inhibition.

$$\%\text{Inhibisi} : \frac{Abs\ control - Abs\ sampel}{Abs\ control} \text{ X } 100\%$$

The average inhibition percentage from three replicates was plotted against the log concentration (µg/mL) and analyzed using non-linear regression fitting (four-parameter logistic model) to determine the IC_{50} value. The IC_{50} results for local duck feet were then compared with the IC_{50} results for ascorbic acid (vitamin C).

Result and Discussion

Characteristics of local duck feet

The proximate analysis of local duck feet consists of protein, ash, and fat content analysis. The ash content of local duck feet is 7.84%, which is higher than that of chicken feet, which is 3.90% (Hashim et al., 2014). The protein content of local duck feet is 9.84%, which is lower than the protein content of chicken feet, which is 17.42% (Liu et al., 2001). The fat content of local duck feet is 7.67%, which is lower than the fat content of chicken feet, which is 8.16%. The pre-treatment stage is important to remove contaminants such as non-collagenous proteins and other impurities such as ash content, which reflects the content of organic minerals such as calcium, phosphate, and magnesium that can affect collagen purity. Suptijah et al. (2018) stated that the pre-treatment stage is carried out to reduce fat and minerals. The difference in characteristics between local duck feet and chicken feet can be influenced by several factors including animal species. Differences in species, habitat, age, feed type, and preparation techniques can cause differences in the chemical composition of various samples (Sukma et al., 2022).

Characteristics collagen

Analysis rendemen

The results of the analysis show that the addition of bromelain enzyme has a significant effect ($P<0.05$) on collagen yield, where the higher the enzyme concentration used, the higher the yield produced. The highest yield was obtained at a bromelain concentration of 0.9%, while the lowest was at 0%. This increase in yield occurs because bromelain is able to hydrolyze the telopeptide region a non-helical region with many cross-links making collagen more soluble. These results are in line with the statement by Putri et al. (2024) that enzymatic treatment can increase collagen solubility and yield. Agrawal et al. (2022) stated that the breaking of bonds in telopeptides increases collagen solubility, which leads to an increase in yield.

The duration of soaking also had a significant effect ($P<0.05$) on the yield produced. Soaking for 24 hours produced a higher yield than soaking for 12 and 18 hours. The longer the soaking time, the more extracellular matrix (ECM) components-such as proteoglycans, lipids, and non-collagen components are degraded, making collagen easier to release. These results are consistent with the statement by Shaik et al. (2023) that an increase in collagen yield can occur as the duration of soaking increases. This increase is due to the breakdown of telopeptides and cross-links by enzymes, making collagen more soluble.

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Protein concentration analysis

The results of the statistical analysis shows the difference in the addition of bromelain enzyme has a significant effect ($P<0.05$) on protein concentration. The amount of bromelain enzyme added can increase the protein concentration of local duck feet collagen. As the concentration of bromelain increases, the enzyme activity in breaking peptide bonds in collagen tissue also increases. High enzyme levels accelerate the hydrolysis process, resulting in the release of more protein molecules into the solution. Amanah et al. (2025) stated that the use of bromelain enzyme in the extraction of collagen from hybrid duck feet increases the hydrolysis of peptide bonds, so that more soluble proteins are released into the medium.

The statistical analysis results show that the difference in soaking time has a significant effect ($P<0.05$) on protein concentration. Longer soaking times provide sufficient time for acids and enzymes to hydrolyze collagen tissue, thereby increasing protein release. Based on research conducted by Kim et al. (2012), which states that soaking conditions (pH and duration) affect the level of swelling, weight gain during hydration, and extraction yield. Soaking at extreme pH (acidic or alkaline) accelerates tissue disintegration and increases protein/collagen release, but the effect is only optimal up to a certain threshold.

The interaction between the amount of enzyme added and the soaking duration had a significant effect ($P<0.05$) on protein concentration. Increasing the amount of bromelain and extending the soaking duration resulted in higher protein concentrations in the discarded duck-feet collagen. Based on research conducted by Susanto et al., (2018), data shows that variations in papain enzyme concentration and immersion time have a significant effect ($P<0.05$) on soluble protein concentration. The higher the enzyme concentration used and the longer the hydrolysis time, the greater the amount of protein released from the chicken foot collagen tissue.

Molecular weight analysis with SDS-PAGE

Local duck foot collagen supplemented with bromelain enzyme shows three main bands, namely β, α1, and α2. Fawzya et al. (2016) stated that type I collagen is composed of α1 and α2 chains that associate to form a triple helix structure. Based on this literature, collagen from local duck feet can be categorized as type I collagen. Local duck foot collagen samples with 0%; 0.3%; 0.6%; and 0.9% had molecular weights in the β band ranging from 207 to 229.89 kDa, the α1 band ranging from 130.45 to 147.90 kDa, and the α2 band ranging from 116.12 to 147.90 kDa. The analysis results are in accordance with Jafari et al. (2020), who stated that the β band in type I collagen has a molecular weight between 200 and 250 kDa, while α1 and α2 have a molecular weight between 120 and 150 kDa. Based on these results, it can be concluded that local duck feet were identified as type I collagen due to the presence of β, α1, and α2 chains. Astiana et al. (2016) stated that the smaller the protein molecule, the faster it will pass through the gel, resulting in a longer travel distance.

Functional group analysis with FTIR

Functional group analysis on local duck foot collagen with the lowest characteristics based on yield and collagen concentration, symbolized as (A1), and the best characteristics based on yield and protein concentration, symbolized as (D3), yield results where the amide A spectrum peak was at 3330.84 cm^{-1} to 3337.26 cm^{-1}. The shift to higher wave numbers shows an increase in hydrogen bond interactions, which indicates that the collagen structure has become more stable. Jafari et al. (2020) stated that amide A is a functional group related to N-H stretching vibrations N-H group located at wavenumbers 3300 to 3440^{-1}. The amide B spectrum peaks in local duck foot collagen A1 and D3 are at 2923.80 cm^{-1} to 2927.61 cm^{-1}. The shift in wavenumber indicates changes in the chemical environment of the protein side chains caused by increased

enzymatic activity that aids in the release of collagen from connective tissue. The peak of the amide I spectrum in local duck foot collagen A1 and D3 is at a wavelength of 1628.75 cm^{-1} to 1656.36 cm^{-1}. The wavelength shift indicates that the triple helix structure of collagen is better formed in collagen D3. Kim et al. (2016) found that amide I was found at a wavelength of 1600 cm^{-1} to 1700 cm^{-1}. The peak of the amide II spectrum in local duck foot collagen A1 and D3 is at a wavelength of 1545.44 cm^{-1} to 1553.21 cm^{-1}. Prajaputra et al. (2025) found that amide II is present at wavenumbers ranging from 1545 cm^{-1} to 1600 cm^{-1}. The peak of the amide III spectrum in local duck foot collagen A1 and D3 is at wavenumbers ranging from 1236.64 cm^{-1} to 1239.18 cm^{-1}. Dhakal et al. (2018) found that amide III can be found at a wavelength of 1200 cm-1 to 1300 cm-1, which is related to the triple helix structure of collagen. The spectrum in the range of 1200 cm^{-1}

Thernal stability analysis with DSC

DSC analysis results show that sample D3 has a higher endothermic peak than sample A1, indicating better thermal stability of collagen. The thermograms of both samples show two peaks, namely the glass transition peak and the melting point peak (Tmax). A high denaturation temperature generally indicates better collagen quality due to the content of hydroxyproline and proline amino acids, which can stabilize the polypeptide structure. Safithri et al., 2020; Agustin et al., 2023; Ahmad & Benjakul, 2010 explain that the high denaturation temperature is influenced by amino acid content and extraction method, where acid-based extraction tends to produce lower thermal stability. Overall, the higher endothermic peak in sample D3 indicates that the collagen is more stable against heat and therefore more resistant for use in product applications.

Degree of collagen hydrolysis

Collagen hydrolysis produces a DH of 85.95%. A high DH value indicates that protease enzymes effectively break collagen chains into short peptides. The smaller the peptide size, the greater the antioxidant activity due to an increase in the availability of proton donor functional groups (Ranathunga et al., 2006).

Antioxidant bioactive activity of peptide with DPPH

Based on the results of the study, it is known that the IC_{50} value of local duck foot collagen hydrolysate is 98.07 µg/mL. The results are in accordance with the study by Prastyo et al. (2020), which found that the best IC_{50} value of tilapia skin collagen hydrolysate was 93.32 µg/mL with a hydrolysis time of 120 minutes. The IC_{50} value is the concentration of a sample that can inhibit 50% of DPPH free radicals. The IC_{50} value is often used as a parameter for antioxidant activity, where a smaller IC_{50} value indicates stronger antioxidant activity of a compound. To evaluate the IC_{50} results of collagen hydrolysate, the results of this study were compared with the IC_{50} results for ascorbic acid (vitamin C). Based on the results of the study, the IC_{50} value of ascorbic acid was 10.32 (µg/mL). The IC_{50} value of ascorbic acid was still lower than the IC_{50} value of local duck foot collagen hydrolysate. This means that ascorbic acid has higher antioxidant activity than local duck foot collagen hydrolysate.

Conclusion

Based on the results of research that has been conducted, it is known that local duck feet can be used as a high-potential source of collagen after undergoing enzymatic hydrolysis. The collagen hydrolysate produced has been proven to contain bioactive peptides with strong antioxidant properties, as indicated by an IC50 value of 98.07 µg/mL. This proves that the utilization of local duck feet waste not only increases the added value of livestock by-products but also provides opportunities for development as a natural antioxidant in functional food products and health applications.

Conflict of interest

No potential conflict of interest relevant to this article was reported. All authors have agreed with the contents o

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This may give an appreciation to Rifqi who help in research and report writing.

Author's contribution

The authors confirm their contribution to the paper as follows: study conception and design: SMD, YE, MZA; data collection: SMD; analysis and interpretation of results: SMD; draft manuscript preparation: SMD.

Ethics approval

This article does not involve animal subjects, so ethical approval for animal studies is not necessary in the present study.

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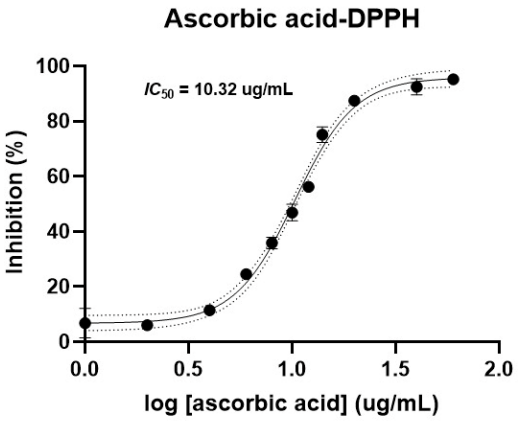

Figure 1. Grafik IC_{50} Ascorbic acid

Table 1. Research design phase 1.

Soaking duration (Hours)	Addition of bromelain enzyme (%) (w/w)			
	0	0,3	0,6	0,9
12	A1	B1	C1	D1
18	A2	B2	C2	D2
24	A3	B3	C3	D3

Table 2. Chemical composition of local duck feet

Parameters	Composition (%)
Ash content	7,84 ± 0,6
Protein	9,84 ± 0,3
Lipid	7,67 ± 0,1

Table 3. Yield of local duck feet (%)

Duration (Hours)	Addition of bromelain enzyme (%)				Average
	0	0,3	0,6	0,9	
12	2,28 ±0,3^{a}	5,70 ±0,7^{bcd}	7,97 ±1,6^{d}	12,91 ±2,5^{e}	7,21 ±4,2^{x}
18	3,71 ±0,5^{ab}	5,92 ±1,0^{bcd}	7,24 ±0,9^{cd}	13,41 ±2,4^{e}	7,57 ±3,9^{x}
24	4,69 ±0,6^{abc}	8,72 ±1,4^{d}	14,81 ±3,0^{e}	20,15 ±2,3^{f}	12,09 ±6,3^{y}
Rerata	3,56 ±1,1^{m}	6,78 ±1,7^{n}	10,01 ±4,0^{o}	15,49 ±4,0^{p}	

Description:

^{abcdef} Different superscripts on the same row and column indicate a significant difference (P<0,05)
^{xy} Superscripts on the same line indicate significant differences in the mean soaking times
^{mnop} Superscripts in the same column indicate significant differences in the mean bromelain enzyme concentrations

Table 4. Concentration of collagen protein in local duck feet (µg/mL)

Duration (Hours)	Addition of bromelain enzyme (%)				Average
	0	0,3	0,6	0,9	
12	160,40 ±0,1^{a}	199,47±0,05^{d}	203,87±0,7^{f}	214,10±0,1^{h}	194,46±21,2^{x}
18	168,20±0,2^{b}	199,67±0,7^{d}	204,37±0,5^{f}	212,97±0,4^{g}	196,30±17,6^{y}
24	182,87±0,2^{c}	203,07±0,05^{e}	213,80±0,5^{h}	220,47±0,4^{i}	205,05±14,8^{z}
Average	170,49±9,8^{m}	200,73±1,7^{n}	207,34±4,8^{o}	215,84±3,4^{p}	
Comersial collagen tipe I *bovine achilles* tendon				17,5 ± 1,38	

Description:

^{abcdefghi} Different superscripts on the same row and column indicate a significant difference (P<0,05)
^{xyz} Superscripts on the same line indicate significant differences in the mean soaking times.
^{mnop} Superscripts in the same column indicate significant differences in the mean bromelain enzyme concentrations.

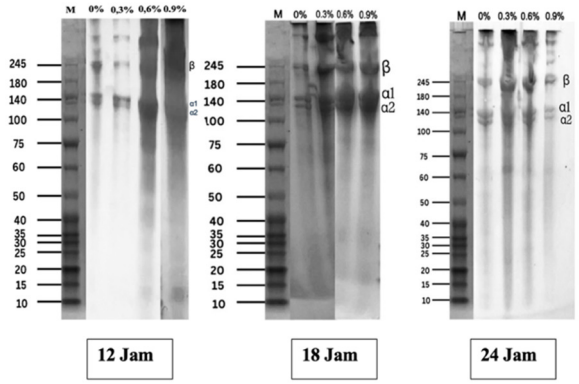

Figure 2. SDS-PAGE Local duck feet collagen
M: Marker

Teble 5. SDS-PAGE analysis

Soaking duration (Hours)	Addition of bromelain enzyme (%)	Band(kDa)		
		β	α1	α2
12 jam	0	224,32	146,10	132,87
	0,3	224,32	147,90	128,86
	0,6	222,95	130,45	116,12
	0,9	224,32	134,92	123,07
	0	229,89	146,55	137,42
	0,3	218,90	146,55	130,05

18 jam	0,6	224,32	137,42	133,28
	0,9	225,70	136,58	132,46
	0	218,23	134,09	143,88
	0,3	207,79	133,28	141,69
24 jam	0,6	211,64	134,92	141,69
	0,9	228,49	132,87	147,90

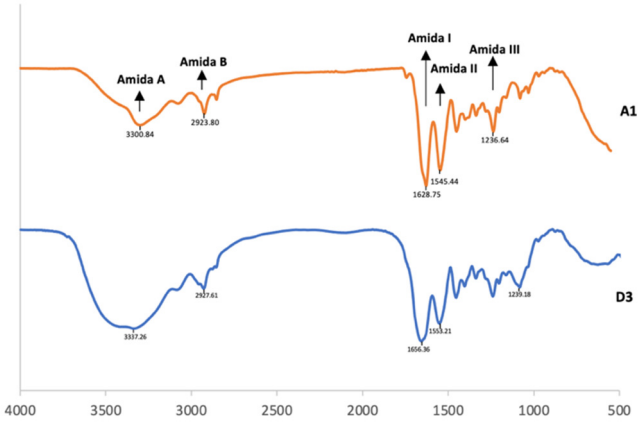

Figure 3. FTIR spectrum of local duck feet collagen
A1:0% 12 hours dan D3: 0,9% 24 hours

Table 5. Peak position of the FTIR spectrum local duck feet collagen

Peak	Wave number (cm^{-1})	
	0%	0,9%
Amida A	3330,84	3337,26
Amida B	2923,80	2927,61
Amida I	1628,75	1656,36
Amida II	1545,44	1553,21
Amida III	1236,64	1239,18

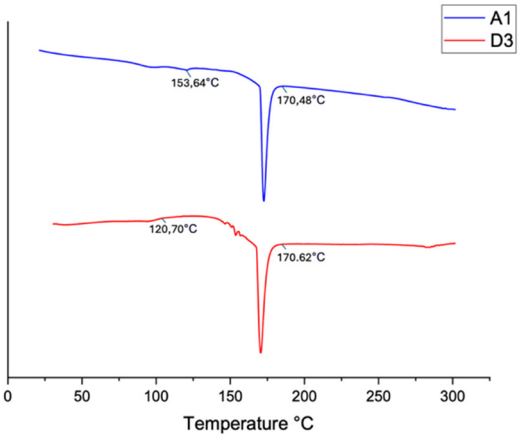

Figure 4. Thermogram of local duck feet collagen
(A1) 0% 12 hours dan (D3) 0,9% 24 hours

Tabel 7. Contrentation of free amino acids in hydrolyzed local duck feet collagen

Free amino acid concentration in collagen (µg/mL)	Free amino acid concentration in hydrolyzed collagen (µg/mL)
29,91	537,22

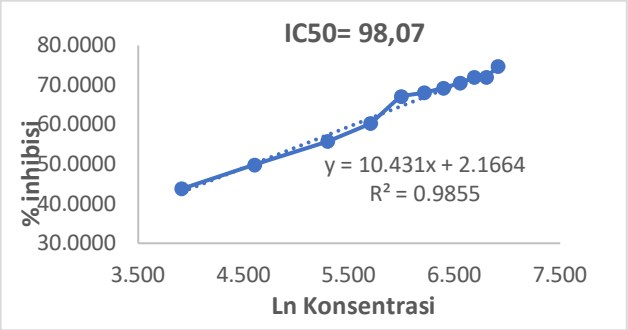

Figure 5. Graph of IC_{50} result for local duck feeet collagen hydrolysate

Table 8. IC_{50} hydrolysate collagen from local duck and Ascorbic acid

IC_{50} Hydrolysate collagen (µg/mL)	IC_{50} Ascorbic acid (µg/mL)
98,07	10,32

