Activity And Kinetics of α-Glucosidase Inhibition by Collagen Hydrolysate From Thunnus albacares Bone

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Abstract. Controlling of postprandial hyperglycemia using α-glucosidase inhibitors is one of the therapeutic approaches for the treatment of type 2 diabetes. Collagen hydrolysate, as an α-glucosidase inhibitor has not been reported. Therefore, This study intends to evaluate the activity and kinetics of inhibition of collagen hydrolysate against the α-glucosidase enzyme. Collagen hydrolysate was prepared from Thunnus albacares bone enzymatically using bacterial collagenase at hydrolysis times (0; 0.5; 1; 2; 3; 4; 5 and 6 h). The type of inhibition was determined by enzyme kinetics analysis. Collagen hydrolysate obtained at hydrolysis time of 1 h has the highest inhibitory activity of 24.47 %. Based on enzyme kinetics analysis, collagen hydrolysate showed a show a type of competitive inhibition. These results revealed that collagen hydrolysate has the potential as a natural antidiabetic in controlling blood glucose levels in type 2 diabetes.

1. Introduction

Diabetes mellitus is a chronic metabolic disease characterized by high levels of glucose in the blood (hyperglycemia) because the body lacks insulin or insulin resistance. Diabetes mellitus is categorized as a global health problem because of the high number of patients with diabetes and its complications being the main cause of premature death in most countries. Especially in type 2 diabetes accounts for around 87-91 % of the total diabetic patients wherein 2015, it reached 318 and is predicted in 2040 to be 481 million [1].

One of the therapeutic approaches for the treatment of type 2 diabetes is controlling postprandial hyperglycemia through inhibition of the α-glucosidase enzyme in the digestive organs [2]. The α-glucosidase enzyme plays a role in breaking down more complex carbohydrates into glucose. Inhibition of this enzyme will reduce the breakdown of carbohydrates causing less glucose absorption and consequently, blood glucose levels do not increase. Currently, oral drugs available as α-glucosidase inhibitors, including acarbose and miglitol cause several side effects, including stomach discomfort, bloating, and diarrhea [3]. Therefore, it is necessary to find an α-glucosidase inhibitor from natural sources that can effectively to treat diabetes.

Collagen hydrolysates have been proposed for the treatment of type 2 diabetes. Oral administration to diabetic patients significantly reduces blood glucose levels, improve insulin sensitivity, and secretion without side effects [4]. Collagen hydrolysate is an interesting research subject for the development of diabetes drugs because of its good penetrating power, high biocompatibility, non-toxic for the body and good bioactivity including antihypertensive, anti-inflammatory and antioxidant capacity which contribute to the prevention or reduction of diabetes complications [5, 6, 7, 8].

Currently, the effects of hydrolysate collagen in managing type 2 diabetes and its complications have been studied both in animal models and directly in diabetic patients related to reduced levels of blood glucose, cholesterol, and fatty acids, inflammation, increased insulin sensitivity, insulin secretion and glucose tolerance [4, 9]. However, there has been no report of its effect in inhibiting the activity of the α-glucosidase enzyme. There is an assumption that free peptides and amino acids in collagen hydrolysate...
can interact or bind to the active site of the enzyme which causes the breakdown of carbohydrates to glucose to be inhibited and thus prevents postprandial hyperglycemia.

Collagen hydrolysate can be obtained from the collagen of fish skin and bone by enzymatic hydrolysis using collagenase. Enzymatic hydrolysis is widely applied to prepare bioactive collagen hydrolysates. Peptides that are present in the inactive form in the peptide chain are activated after the hydrolysis process [10, 11]. In this study, we reported the preparation of collagen hydrolysate from yellowfin tuna (*Thunnus albacares*) bone enzymatically using bacterial collagenase. Collagen hydrolysate obtained was evaluated for activity and kinetics of inhibition against the α-glucosidase enzyme.

2. Experimental

2.1 Materials and Method

Yellowfin tuna (*Thunnus albacares*) bone was cleansed from remaining of meat, fat, and blood. Then it was cut into small pieces, sealed in plastic bags and stored in a freezer until further use.

2.3 Extraction of Collagen

Collagen from *T. albacares* bone was extracted based on a modification of Baehaki method [12]. To remove non-collagenous compounds, fish bone was soaked in 0.1 M NaOH with a ratio of 1:10 (w/v) followed by stirring for six hours. The solution was replaced every two hours, then washed with cold distilled water until neutral pH. To hydrolysis, it was soaked in 1.5 % acetic acid with a ratio of 1:2 (w/v) for 24 h and washed with cold distilled water until neutral pH. Collagen was extracted using aquadest with a ratio of 2:1 (w/v) for 3 h at 45 °C. Collagen extract was filtered and stored in a freezer until further use.

2.4 Preparation of Collagen Hydrolysates

Collagenase from *Bacillus* sp. 6-2 was reacted with 1 mM of CaCl$_2$ with a ratio of 10:1 (v/v) at pH 7.0 and 40 °C for 10 min, then used to hydrolyze 10 % collagen solution (2.2 mg/mL) with an enzyme-substrate ratio of 1:10 (v/v). Hydrolysis was formed at pH 7.0 and 40 °C for 0; 0.5; 1; 2; 3; 4; 5 and 6 h. The enzyme was inactivated by heating in boiling water for 5 min and centrifuged for 10 min at 10.000 rpm and 4 °C. The supernatant was stored in a freezer for further analysis.

2.5 Determination of Soluble Protein Concentration

Soluble protein fraction was obtained by reacting collagen hydrolysate and 20 % TCA following a procedure of Hoyle and Merritt [13] with modification. A total of 0.5 mL of collagen hydrolysate was reacted with 0.5 mL of 20 % TCA at 4 °C for 30 min. The mixture was centrifuged at 3500 rpm, 10 °C for 20 min. The soluble protein concentration of the supernatant was determined according to the Lowry method, with bovine serum albumin (BSA) as standard [14].

2.6 Assay for α-Glucosidase Inhibition

Inhibition activity of collagen hydrolysate against α-glucosidase was determined using the spectrophotometric method following a procedure of Natsir et al. (2018) with modification. Collagen hydrolysate and acarbose were prepared in a concentration of 1000 ppm [15]. A total of 0.2 mL of collagen hydrolysate was added to a tube containing 5 mL of 0.1 M phosphate buffer (PH 7) and 0.2 mL of the α-glucosidase enzyme. This solution was pre-incubated at 37 °C for 10 min and added 0.5 mL of 10 mM PNPG substrate, then further incubated at 37 °C for 20 min. A total of 2 mL of the mixture was
taken and reacted with 8 mL of 0.1 M Na₂CO₃. The absorbance of the solution was measured using a spectrophotometer at λ 400 nm. Enzyme activity was calculated by the formula:

\[
\text{Enzyme activity (U/mL)} = \frac{(A_{\text{sample}} - A_{\text{blanko}}) \times \text{Total volume analyzed} \times \text{Volume (enzyme + substrate + buffer)}}{\text{Molar extinction coefficients} \times \text{Incubation time} \times \text{Mixture volume} \times \text{Enzyme volume}}
\]

Where A was absorbance and value of molar extinction coefficients was 18.3.

2.7 Assay for Kinetics of α-glucosidase Inhibition

Kinetics assay was carried out by determination of enzyme activity at substrate concentrations varied (5; 7.5; 10; and 12.5 Mm) with or without the addition of samples. The sample used is collagen hydrolysate with the highest inhibitory activity. The type of inhibition of collagen hydrolysate on α-glucosidase activity was determined by analysis of Lineweaver-Burk plot using Michaelis-Menten kinetics.

3. Results and Discussion

3.1 Soluble Protein Concentration

Soluble protein concentration (SPC) shows the number of peptides and free amino acids contained in the hydrolysate sample. During hydrolysis, there is the release of a number of peptides and free amino acids because of the breakdown of peptide bonds by enzymes. In this study, the soluble protein was obtained by the deposition method using TCA. The principle of this method is the separation of soluble and insoluble protein fractions in the hydrolysate sample after centrifugation [13]. This method is relatively simple, inexpensive, and the analysis process is faster than another method.

SPC of collagen hydrolysate obtained at different hydrolysis time was shown in Figure 1. SPC at 0 h (without the addition of enzymes) was only about 0.065 mg/mL, much lower than the SPC obtained by the addition of enzymes. The presence of soluble proteins at 0 h might be caused by hydrolysis in a small part of the structure of collagen during extraction at 45 °C. According to Gómez-Guillén et al. (2009) [16], collagen is an unstable protein to thermal conditions and extraction at high temperatures will cause hydrolysis which converts the helical structure of collagen into a random coil of gelatine.

![Figure 1: The soluble protein concentration of collagen hydrolysate](image)

SPC increased sharply to 0.420 mg/mL after at 0.5 h hydrolysis with collagenase, then increased slowly until it reached the optimum at 4 hours with a value of 0.530 mg/mL. This result showed that during hydrolysis, collagenase actively converts insoluble proteins to soluble proteins through the breakdown of collagen polypeptide chains into collagen peptides. Increase hydrolysis will cause further breakdown of larger peptides into smaller peptides and free amino acids, and therefore, the SPC value increases. This accorded with Mutamimah et al. (2018), who reported that SPC of protein hydrolysate prepared from tuna eye protein using papain 0.15 % increases with increasing hydrolys time [17]. The increase in SPC during the hydrolysis time indicates that the hydrolysis process is good.
3.2 Activity of α-Glucosidase Inhibition

Inhibitory activity of α-glucosidase by collagen hydrolysate obtained at different hydrolysis time was shown in Table 1.

| Hydrolysis Time (hour) | α-Glucosidase Activity (mU/mL) | % Inhibition |
|------------------------|-------------------------------|-------------|
| Negative control       | 57.62                         | 0           |
| Positive control       | 20.15                         | 65.03       |
| 0                      | 49.57                         | 13.98       |
| 0.5                    | 47.55                         | 17.47       |
| 1                      | 43.52                         | 24.47       |
| 2                      | 47.15                         | 18.17       |
| 3                      | 49.17                         | 14.68       |
| 4                      | 51.58                         | 10.48       |
| 5                      | 53.60                         | 6.98        |
| 6                      | 53.20                         | 7.68        |

Inhibitory activity of samples without enzyme addition (0 h) increased after hydrolysis for 0.5 h, from 13.98 % to 17.47 %. This proved that enzymatic hydrolysis using collagenase could increase bioactivity because of the release of specific peptide fragments from collagen polypeptide chains, which can inhibit α-glucosidase activity. Inhibitory activity increased with increasing SPC, but after 1 h it decreased. This is most likely because, during hydrolysis, the active peptide in collagen hydrolysate is further degraded into small peptides and free amino acids with lower activity.

The highest inhibitory activity was obtained at 1 h hydrolysis of 24.47 %. This result is lower than positive control acarbose (65.03 %). However, this result first reports the ability of collagen hydrolysate to reduce blood glucose levels through inhibition of α-glucosidase. Therefore, it has the potential as a natural inhibitor for controlling postprandial hyperglycemia. The low activity obtained is because the tested sample is still a crude fraction (origin hydrolysate). Inhibitory activity of samples can be increased through the separation of bioactive components. This statement was supported by Ramadhan et al. (2018), who reported that the separation of peptide components from Andrias davi dianus protein hydrolysate caused an increase in activity (above 50 %), even 4 times more active than the origin hydrolysate [18]. These result suggested for separation of bioactive peptides in this study.

In this study, SPC value and inhibitory activity did not show correlation; this is because many factors that influence the inhibitory activity of collagen hydrolysate. Besides the size of the peptide, it is also influenced by the type and sequence of amino acids in the peptide [19, 18]. Inhibitory activity of collagen peptides is suspected because of the high of residual amino acids such as glycine, proline, and alanine, which are the main amino acids of collagen [20]. This is as reported by Ramadhan et al. (2018), that glycine, proline, and alanine were found to contribute to the inhibitory activity of peptides isolated from Andrias davi dianus protein hydrolysate in a sequence of Pro-Gly-Gly-Pro and Ser-Ala-Ala-Pro [18]. The presence of high quantities of glycine and proline showed strong inhibitory activity. Zhang et al. (2015) also found that glycine, proline, and alanine contributed to the inhibitory activity of silkworm cocoons peptides [21]. Besides, Matsui et al. (1999) reported that tyrosine, proline, and leucine in peptide sequences are important element for α-glucosidase inhibitory activity [19].

3.3 Kinetics Analysis of α-Glucosidase Inhibition

Determination of the type of inhibition was carried out by analysis of Lineweaver-Burk plot to obtain the enzyme constant (Km) and maximum rate (Vmax) values, which was calculated based on the regression equation, \( y = ax + b \), with \( 1/[S] \) as the X-axis, and \( 1/V \) as the Y-axis. The Km and Vmax values were shown in Table 2.
Table 2. Kinetics analysis of α-glucosidase inhibition by collagen hydrolysate

|                      | Without Sample | Sample |
|----------------------|----------------|--------|
| Km (mM)              | 9.8            | 17     |
| Vmax (mM/men)        | 200            | 200    |

Figure 2. Lineweaver-Burk plot of kinetic analysis of α-glucosidase inhibition by collagen hydrolysate

Based on the Lineweaver-Burk double plots, the two straight lines obtained after linear regression intersects at the same point on the Y-axis showed that Vmax in the reaction system remains after the addition of addition it also causes a significant increase in Km values from 9.8 to 17. The inhibition mechanisms, which increases Km and Vmax values, remains a competitive inhibition mechanism [22].

The Km value increased after the presence of the sample showed that the affinity or attraction of the enzyme for the sample was higher than for substrate. As a competitive inhibitor, the collagen peptides in the sample can bind to free enzymes to result in reducing the affinity between enzyme and substrate, thereby the Km value increases. The inhibiting mechanism in this study is in accordance with that reported by Matsui et al. (1999), who found that the Tir-Tir-Pro-Leu peptide acts as an α-glucosidase inhibitor through interaction or binding on the active site of the enzyme [19]. Peptides with leucine residues at the C-terminal end and tyrosine at the N-terminal end were found to be most important for binding or interacting with the active side of the α-glucosidase enzyme. (Zhang et al., 2015) also found four peptides from silkworm cocoons, which had the same inhibitory mechanism [21].

4. Conclusion
Collagen hydrolysate prepared from Thunnus albacares bone enzymatically using bacterial collagenase with the hydrolysis time of 1 h showed the highest inhibitory activity of 24.47 %. Enzyme kinetics analysis revealed that collagen hydrolysate is a competitive inhibitor. These results suggested that bioactive components in collagen hydrolysate of Thunnus albacares bone have the potential as a natural inhibitor in controlling blood glucose levels in type 2 diabetes. Separation and identification of bioactive peptides from collagen hydrolysate are needed to improve its activity as α-glucosidase inhibitors.
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