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by Yoni Atma

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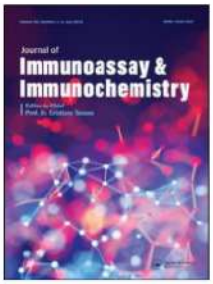

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The proportion–ratio on *dipeptidyl aminopeptidase*-4 (DP-4) inhibition by gelatin compared to synthetic sitagliptin

Yoni Atma[a], Hanifah Nuryani Lioe[b], Endang Prangdimurti[b], Hermawan Seftiono[a], Moh Taufik[a], Dita Fitriani[a], and Apon Zaenal Mustopa[c]

[a]Department of Food Science and Technology, Faculty of Bioindustry, Universitas Trilogi, Kalibata, Jakarta, Indonesia; [b]Department of Food Science and Technology, Faculty of Agricultural Engineering and Technology, Bogor Agricultural University, Bogor, West Java, Indonesia; [c]Division of Molecular Biology, Research Center for Biotechnology, Indonesian Institute of Science (LIPI), Cibinong, Bogor, West Java, Indonesia

ABSTRACT

The current study aims to determine the inhibition activity gelatin against *dipeptidyl aminopeptidase* 4 (DP-4). Two commercial gelatins, i.e., bovine and fish skin gelatin and one extracted (in our laboratory) gelatin, i.e., fish bone gelatin were selected for analysis. Each gelatin have same protein pattern (75–245 kDa) on sodium dodecyl sulfate polyacrylamide gel electrophoresis with mean of protein concentration of 1.72 mg/mL. The inhibition activity was measured on the capacity to inhibit DP-4 by using *Gly-Pro-p-nitroanilide* as their substrate. The sitagliptin was used as standard comparison. Based on the percent inhibition, gelatin has been shown to be the prospective DP-4 inhibitor.

KEYWORDS

Dipeptidyl peptidase IV (DPP-4); gelatin; incretin; inhibitory activity; diabetes mellitus type 2

Introduction

Dipeptidyl aminopeptidase (DP-4) or *dipeptidyl peptidase* 4 (DPP-4) is a protease that degrades some hormones, including incretin. Incretin, which is formed by *glucose-dependent insulinotropic polypeptide* (GIP) and *glucagon-like peptide*-1 (GLP-1), has a short half-life (only 1–2 min) because of DP-4 activity. The degradation of GIP and GLP-1 causes the loss of incretin.[1] Incretin, however, has an important role regarding blood glucose homeostasis, and it was promising therapeutic target in type 2 diabetic therapy.[2] Therefore, recently, many studies on seeking and characterizing of DP-4 inhibitor have been growing rapidly.

Some of DP-4 inhibitors has been patented and commercialized including synthetic substances such as sitagliptin, saxagliptin, and linagliptin.[3] Unfortunately, the synthetic DP-4 inhibitors potentially have side effects that lead to other diseases.[4] So that, research in looking for DP-4 inhibitor

CONTACT Yoni Atma yoniatma@trilogi.ac.id Department of Food Science and Technology, Faculty of Bioindustry, Universitas Trilogi, Kalibata, Jakarta 12760, Indonesia

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from natural sources pay close attention. The natural compound has been studied as DP-4 inhibitor including the substance of plant-based extract[5,6]; however, it also dominated food-based protein such as milk protein, beef, cheese, egg protein as well as fish protein and gelatin.[7] Some studies were done for investigating the inhibitory activity of gelatin and their hydrolysate against DP-4 covering porcine, bovine, and fish gelatin.[4,8,9] The application of porcine gelatin as source DP-4 inhibitor has been limited because this source is prohibited in Islam and Judaism. Fortunately, most of bovine and fish gelatin which was analyzed as DP-4 inhibitor were derived from the by-product. Fish processing by-product has been most potential as alternative source for gelatin extraction.[10] For instance, the research and application about bovine and fish-based gelatin-related DP-4 inhibitor would be promising in the future. Even less, gelatin has been widely used for a long time as a safe and unique ingredient in food and pharmaceutical industries.[11]

The aim of this research was to determine the percent inhibition of bovine, fish skin, and fish bone gelatin against DP-4 through their ratio inhibition compared to synthetic sitagliptin. In the previous studies, the quantified inhibitory activity of gelatin and another food-based protein against DP-4 enzymes do not use sitagliptin as standard, but diprotin A.[4,8,12] Actually, sitagliptin is a well-known synthetic DP-4 inhibitor in the market.[3,13] The seeking for DP-4 inhibitors from natural substances and gelatin is certainly intended to replacing this synthetics medicine.

Experimental method

Gelatin preparation

Two commercial gelatins were used in this study, i.e., bovine gelatin (technical gelatin, no brand) and fish skin gelatin (progel, Ho Chi Minh, Vietnam), while fish bone gelatin extracted from the bone of *Pangasius catfish* by pretreatment and main extraction was based on Pertiwi et al.[14] In the pretreatment, the bone was soaked in 1% citric acid solution for 48 hr to obtain the leach bone (ossein). Then, the ossein was separated with the solvent through centrifugation (5000 g, 10 min), then it was washed with aquadest until the pH became neutral. Later, the main extraction was carried out by soaking the ossien in aquadest for 48 hr at 75° C. The clear extract of gelatin was collected by filtration using filter paper. At last, the extract was dried by food dehydrator (Excalibur, Sacramento, USA) for 8 hr at 55°C. The gelatin was dissolved with distillated water in concentration of 25 mg/mL (m/v), stored at 4°C, and thawed before further analysis.

Vertical electrophoresis

Vertical electrophoresis was used as confirmation test regarding of gelatin existence in the solution. It was performed using *sodium dodecyl sulfate*

polyacrylamide gel electrophoresis (SDS-PAGE). The SDS-PAGE was carried out by discontinues Tris/HCl/glycine buffer system.[15] Each gelatin was diluted in buffer containing 0.5 M Tris-HCl pH 6.8 with 3% (w/v) sodium dodecyl sulfate (SDS), 30% (v/v) glycerol, and 15% (v/v) β-mercaptoethanol at ratio 1:1 (v/v). A 12% separating gel and 3.9% stacking gel were used. Then, a 20 µL sample and protein marker (SMOBIO PM 2700, Hsinchu, Taiwan) was run in electrophoresis device (ATTO Page Ace, Tokyo, Japan) at voltage of 50 V for 90 min. After that, the gel from electrophoresis was stained with Coomassie Brilliant Blue R-250 for 24 hr until a clear blue background gel was obtained.

DP-4 inhibition

DP-4 inhibitory activity was determined based on DP-4 activity for using their substrate in the presence of sample. It is interpreted by the capacity of reacted solution to absorb color at visible wavelength.[16] To measure DP-4 inhibition, first, gelatin was added to the solution of *Gly-Pro-p-nitroanilide* (Sigma, St. Louis, MO, USA) 1.59 mM in 100 mM buffer Tris pH 8 in a ratio 1:1 (v/v). This mixed solution was then incubated at 37°C for 10 min. Next, the DPP-4 (Sigma, St. Louis, MO, USA) was diluted using 100 mM buffer Tris pH 8 and pipetted 40 µl DPP-IV (concentration of 0.01 U/mL) was mixed with 80 µl mixing solution that contains sample gelatin and *Gly-Pro-p-nitroanilide*, which was earlier prepared. After that, the solution was gently shaken and reacted at 37°C for 60 min. Then, this reaction was stopped by adding 100 µl of sodium acetate 1 M pH 4. Finally, the absorbance of solution measured using *enzyme-linked immunosorbent assay* (ELISA) Microplate Reader (Multiskan Ex, Champaign, IL, USA) at a wavelength of 405 nm. Sitagliptin (European Pharmacopoeia, Strasbourg, France) with a concentration of 0.0001–0.01 ng/mL was used as standard comparison. The percent of inhibition calculated by setting up absorbance from 0.1 ng/mL sitagliptin as 100% which is multiplied with the another absorbance of inhibition reaction.

Protein quantification

The protein concentration from three sources of gelatin (bovine, fish skin, and fish bone) were quantified using *bicinchoninic acid* (BCA) assay kit (Thermo Fisher, Rockford, IL, USA) by the method described Atma and Ramdhani.[17] Each gelatin solution was pipetting as many as 10 µL, then putted into each well in 96-well plate. After that, 200 µL BCA solution that contains solution A (sodium carbonate, sodium bicarbonate, bicinchoninic acid, sodium tartrate in 0.1 M sodium hydroxide) and solution B (copper (II) sulfate) in ratio 50:1 (v/v) was added. Then, the mixture was mixed and incubated for 30 min at room temperature. The absorbance of sample in BCA solution was measured at 560 nm with ELISA Microplate Reader

(Multiskan Ex, Champaign, IL, USA). In this quantification procedure, the bovine serum albumin in the range of 0–1 mg/mL was used as standard solution.

Data analysis

Data were analyzed using one-way analysis of variance, followed by Tukey's Honestly Significant Different test or Tukey's range test to statistically determine the level of significance difference ($P < .05$) between data.

Result

Gelatin confirmation test

In this study, all three types of gelatin, i.e., bovine, fish skin, and fish bone, appeared at the molecular weight range of 75–245 kDa. It was confirmed as gelatin with protein concentration of 2.19 ± 0.008, 1.70 ± 0.002, and 1.27 ± 0.004 mg/mL, respectively. The electropherogram or SDS-PAGE of the gelatin is presented in Figure 1, while the protein concentration each of gelatin is shown in Table 1. Table 1 also demonstrated the value of percent inhibition divided by protein concentration where the gelatin obtained from the bone of fish was higher compared to that of bovine and fish skin gelatin. The highest percent of inhibition from gelatin was used for determining this value.

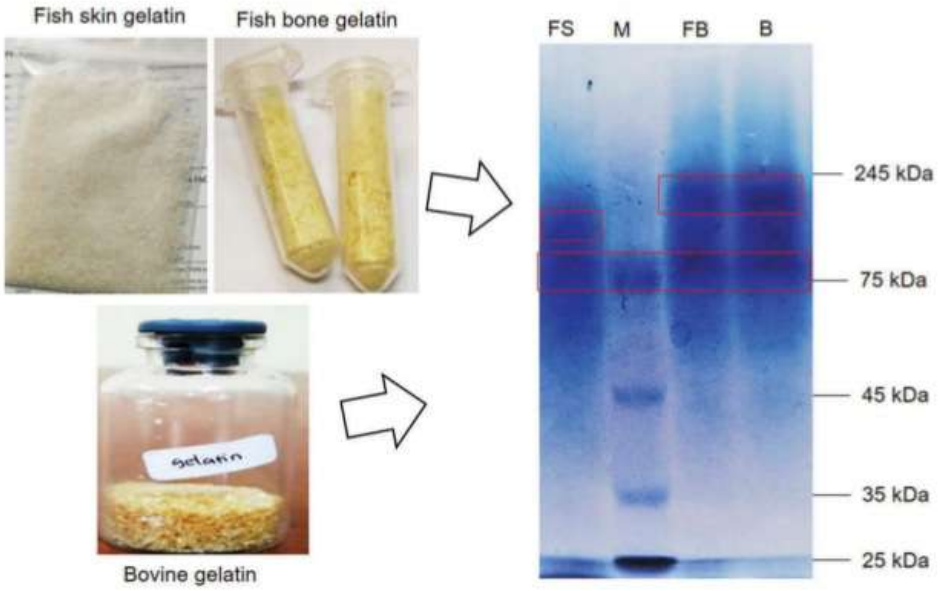

Figure 1. Electroforegram bovine gelatin (B), fish skin (FS), and fish bone (FB) gelatin; M = marker protein.

Table 1. Protein concentration and value of percent inhibition divided protein concentration of gelatin.

Sources of gelatin	Protein concentration (mg/mL)	Percent inhibition divided by protein concentration (% inhibition per mg/mL protein)[a]
Bovine	2.19 ± 0.008[a]	8.58 ± 1.15[b]
Fish Bone	1.27 ± 0.004[c]	17.23 ± 1.21[a]
Fish Skin	1.70 ± 0.002[b]	6.96 ± 0.54[b]

Values in the same row followed by different letters are significant differents ($P < 0.05$)
[a]The highest percent inhibition from gelatin was used for determining the value of percent inhibition divided by protein concentration.

DP-4 inhibition

One of the most used procedures for determining the inhibition capacity of gelatin against DP-4 is through the measurement of DP-4 activity by using *Gly-Pro-p-nitroanilide* as their substrate in the presence and absence of gelatin. The increase in absorbance in the presence of gelatin at visible wavelength on DP-4/substrate interaction increases the inhibitory activity. In this study, sitagliptin was used as positive control. The inhibitory activity performed in percent (%) was quantified based on the absorbance sample gelatin compared to the absorbance of sitagliptin, and then multiplied by 100%. There are three concentrations of sitagliptin that was used as control in the range of 0.0001–0.01 ng/mL. Previously, Nongonierma and FitzGerald was used 0.006 and 0.03 ng/mL sitagliptin, regarding the study on inhibitory properties of whey protein against DP-4 enzyme.[18] Overall, the percent of inhibition reduced by increasing the standard concentration of sitagliptin. By using 0.0001 ng/mL sitagliptin, the percent of inhibition reached its peak to 21.8%. In this research, the gelatin derived from fish bone was higher in percent of inhibition against DP-4, while the gelatin from fish skin was the lowest (9.8–11.8%). In addition, the percent of inhibition bovine gelatin was 15.6–18.8% where is no significant difference ($P < .05$) was found to correlate in fish bone gelatin, and significant difference was found in fish skin gelatin. The inhibitory activity of the three gelatin sources in this study compared to sitagliptin DP-4 inhibition is presented in Figure 2.

Discussion

SDS-PAGE was used to confirm the presence of gelatin in the prepared solution. The SDS-PAGE was used for protein identification based on their molecular weight. The gelatin is formed by different polypeptides categorized as α-chain, β-chain, and γ-chain gelatin. The α-chain gelatin has molecular weight in the range of 97–140 kDa, β-chain gelatin has molecular weight about 150–250 kDa, and γ-chain gelatin has molecular weight of >250 kDa.[19] Bovine gelatin has molecular weight in the range of 100–200 kDa.[20] The fish bone gelatin also has molecular weight range in 100–200 kDa. In more detail, the molecular weight of fish bone

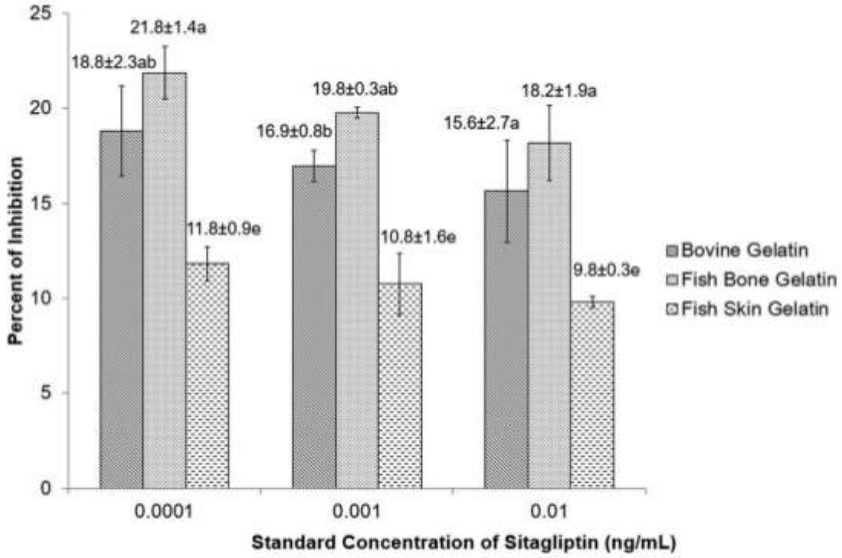

Figure 2. Percent (%) inhibition of bovine, fish bone, and fish skin gelatin against DP-4 by proportion–ratio with sitagliptin at concentration 0.0001–0.01 ng/mL. Different letters indicate significant differences ($P < .05$).

gelatin from Pangasius catfish was ~100–116 kDa, ~150–200 kDa and > 225 kDa[15], from Nila perch was 100–150 kDa[21], and the molecular weight of Channel catfish was ~100 kDa.[22] While the gelatin from skin of fish has molecular weight in the range of 97–130 kDa.[23] Based on these literatures, the gelatin existance was confirmed. In addition, the important thing for seeking the inhibitory activity of bioactive compound is their concentration, besides their activity.[24] Generally, it is expected that low concentration of substance provides high bioactivities. Therefore, in this study, the value of percent inhibitions was also determined by dividing the protein concentration. From Table 1, it was concluded that even though the fish bone gelatin is lower in protein concentration compared to bovine and fish skin, however, it is potentially developed through their activity consideration.

Furthermore, related DP-4 inhibitory activity of bovine and fish-based gelatin. It has been known that DP-4 is serine protease in which postproline cleaving enzyme specifically removes X-proline or X-alanine dipeptide from N-terminus of polypeptide. DP-4 enzyme also degrades the GIP and GLP-1. It produces two substances that build incretin hormone losing their function.[25] Incretin has an important role in maintaining homeostasis of blood glucose associated with type 2 diabetic mellitus (T2DM) prevention and treatment.[2] Consequently, studies on seeking and characterization of DP-4 inhibitor have been growing rapidly; one among them is about gelatin.[4,8,25] Gelatin has been widely used for a long time as a safe and unique ingredient in food and pharmaceutical industries, and their imino

acid (proline+hydroxyproline) composition also makes this colloid fit for DPP-IV inhibition.[4,8,25,26]

The measurement of inhibitory activity against DP-4 enzyme uses sitagliptin as positive control, which was used as inhibitor of DP-4 and used as medicine for T2DM therapy as well as treatment manner. Some synthetic medicines have been used as DP-4 inhibitors, including sitagliptin, saxagliptin, linagliptin, and vildagliptin.[27] Study by Tatosian et al.[3] concluded that treatment of diabetic patient with sitagliptin trough DP-4 inhibition was significantly greater than another such as saxagliptin or vildagliptin administered once daily. Administration of once-daily sitagliptin is similar to that provided by vildagliptin administered twice daily. Sitagliptin categorized as non-substrate like inhibitor for DP-4.[28] In our research paper, inhibitory activity of sitagliptin determined of 100% inhibition because it was standard DP-4 inhibitor.

Implementation of sitagliptin as standard was also carried out by Ekayanti et al. (2018), in which showed that DP-4 inhibitor was quantified from white tea (*Camellia sinensis* (L.) Kuntze) extract. It was found that the white tea extracted using methanol has greatest DP-4 inhibition than hexane and ethyl acetate. The percent inhibition of white tea fraction extracted using methanol, hexane, and ethyl acetate was 50.48, 32.42, and 36.54%, respectively.[5] Another research regarding DP-4 inhibitor using sitagliptin as comparison medicine was also conducted by Riyanti et al. (2016) by using various plants. Based on this research, it was found that leaves of various plants have percent inhibition about 13.94 ± 4.08%–68.98 ± 1.95% against DP-4. The inhibitory activity of semen, herb, stem, and rhozime of herb plant against DP-4 was 6.48 ± 0.32%–71.29 ± 0.33%, 33.52% – 37.03 ± 0.65%, 65.86 ± 1.02%, and 17.12 ± 1.95% respectively.[6]

The percent of inhibition gelatin against DP-4 enzyme was also studied previously. The porcine gelatin has percent inhibition about 9.2%,[8] while the gelatin from salmon skin was about 10%.[25] The gelatin from skin of pacific hake, halibut, nila tipalia, and milkfish has percent inhibition lower than 10%.[4] In addition, fish protein from tuna sauce has inhibitory activity of 9.8%.[12] This inhibitory activity gelatin was increased after hydrolysis and ultrafiltration.[4,8,25] All of those studies used diprotin A as DP-4 inhibitor as standard comparison. The IC_{50} of diprotin A was 24.7 μM,[25] while the IC_{50} of sitagliptin was 18–20 nM.[29]

Regarding their mechanism in human intestinal tract, gelatin is likely hydrolyzed by gastric and intestinal protease. Protein and/or gelatin is hydrolyzed in gastrointestinal route and then absorbed only in a form of free amino acid, di- and tripeptides[26] indeed, so it might become prospective and challeging for the future studies about the peptide derived from gelatin hydrolysis in human digestion tract correlated with their bioactivities as DP-4 inhibitor. It is highly potential if the gelatin

hydrolyzed to become bioactive peptide which directly absorbed in human intestinal ephitellium. Based on previous study was indicated that the collagen-derived peptides have bioactivities after absorption from the digestive tract by in vivo experiment, and several researches have been focused on the effect of oral intake in both animal and human models, which have expressed the excellent absorption and metabolism on Hyp-containing peptides.[30] However, if the peptide cannot be directly absorbed after passing through the human digestive tract, it will be a challege to discover a vehicle transporting the peptide lead up to the human systemic circulation.

Conclusion

Percent inhibition of bovine, fish skin, and fish bone gelatin against DP-4 was 9.8–21.8% using sitagliptin as standard comparison. Each percent inhibition of gelatin was increased if sitagliptin, used as standard comparison, decreased. Among the three gelatin sources, the inhibitory activity of gelatin from fish bone was the highest. It was not significant compared to bovine gelatin; however, it was significantly different when compared to fish skin gelatin. The proportion–ratio of percent inhibition of gelatin in this study was found to be higher when compared to another fish-based protein and gelatin.

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