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Characterization of Fish Skin Hydrolysates Exhibiting Dipeptidyl Peptidase IV Inhibitory Activity

Honoka Miyamoto[1], Erwina Safitri[1], Takeshi Nagai[2], Masataka Saito[1*]

[1] Laboratory of Food Science and Technology, Graduate School of Kagawa Nutrition University, Kagawa Nutrition University, Saitama, Japan, 350-0288
[2] Graduate School of Agricultural Sciences, Yamagata University, Yamagata, Japan, 990-8560

[*]Corresponding author: msaito@eiyo.ac.jp

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ABSTRACT: Dipeptidyl peptidase IV (DPP-IV) inhibitors are antidiabetic drugs that can lower blood sugar levels. There are still few reports on the DPP-IV inhibitory activity of peptides obtained from discarded fish skin. Therefore, we prepared various enzymatic hydrolysates using the skins of six fish species and investigated their DPP-IV inhibitory effects. As a result, it was found that the DPP-IV inhibitory activity of yellowtail hydrolysate by Alcalase was higher than that of other enzymes. In addition, the IC_{50} after ethanol fractionation was found to be lower in yellowtail and eel skin hydrolysate. Amino acid composition analysis showed that the hydrolysate obtained from the skin of the yellowtail contained the highest amount of Gly, followed by Pro, Hyp, and Ala, indicating that it was a peptide derived from type I collagen. Fractionation with ethanol showed that the DPP-IV inhibitory components were contained in the low molecular weight fraction. The artificial digestion test observed no DPP-IV inhibitory activity or average molecular weight change. The DPP-IV inhibitory peptide obtained from fish skin has the potential to be applied as a food material to various food products.

Keywords: Alcalase, collagen hydrolysate, dipeptidyl peptidase IV (DPP-IV), fish skin

INTRODUCTION

Dipeptidyl peptidase IV (DPP-IV) inhibitor is one of the antidiabetic drugs, and by inhibiting DPP-IV existing in the body, it suppresses the decomposition of incretin and sustains insulin secretion (Smushkin *et al.*, 2009). It can consequently suppress the hyperglycemia characteristic of type 2 diabetes (Thoma *et al.*, 2003; Gheni *et al*., 2014). DPP-IV inhibitors maintain blood glucose-dependent insulin action, so there are only a few side effects of conventional drugs, such as weight gain and hypoglycemia. However, there are concerns about side effects such as gastrointestinal side effects, acute pancreatitis, and heart failure (Theodosis *et al*., 2014; Desai *et al*., 2010; Nonaka *et al*., 2008). In recent years, it has been reported that peptides obtained from proteins contained in milk, eggs, walnuts, etc., exhibit DPP-IV inhibitory activity and are attracting attention as food materials that can inhibit DPP-IV without side effects (Jia *et al*., 2020; Nong *et al*., 2020; Kong *et al*., 2021).

More than 80% of the total protein in fish skin discarded as processing residue is composed of type I collagen (Kimura *et al*., 1999; David *et al*., 2020). Type I collagen fulfills the main supporting function not only in fish but also in all vertebrates as the major fibrillar component. Since collagen contains abundant Pro and Hyp residues, collagen-derived peptides may have a high affinity for DPP-IV, which recognizes the Pro residues of peptides (Wang *et al.*, 2021). In addition, since fish skin type I collagen differs in amino acid composition and sequence from that of mammals, the obtained peptides are thought to exhibit different inhibitory activities and properties depending on the fish species (Zhuang *et al*., 2012). DPP-IV inhibitory peptides derived from fish skins such as Atlantic salmon (Jin *et al.*, 2020) and tilapia (Liu *et al.*, 2022) have been recently reported as food materials for human health.

In this study, we investigated the DPP-IV inhibitory effects of peptides produced by various enzymes. We aimed to develop fish skin-derived hydrolysates (peptides) containing DPP-IV inhibitory active ingredients as food materials. In addition, enzymatic hydrolysates were prepared from fish skins of yellowtail, eel, Pacific flounder, mackerel, rainbow trout, and opah,

which are generally considered edible, and peptides exhibiting inhibitory activity were compared.

MATERIALS AND METHOD

Materials

As samples, yellowtail (*Seriola quinqueradiata*), eel (*Anguilla japonica*), Pacific flounder (*Pseudopleuronectes herzensteini*), mackerel (*Scomber australasicus*), opah (*Lampris guttatus*), and rainbow trout (*Oncorhynchus mykiss*) were purchased from the market and used. The opah was provided by the Central Research Institute of Fisheries, Japan Fisheries Research and Education Agency. The wet weights of skins collected from yellowtail, eel, Pacific flounder, mackerel, opah, and rainbow trout were 69.8 g, 52.4 g, 22.4 g, 21.9 g, 19.2 g, and 16.6 g, respectively, and were kept at -40 ℃ until used.

Preparation of dried fish skin and conditions for enzymatic hydrolysis

DPP-IV inhibitory peptide was prepared from yellowtail skin to examine the enzymatic degradation conditions. First, scales and fish meat were carefully removed from the yellowtail fillet, and the obtained dermis was cut into 5 mm squares. Then, 10 times the amount of hexane-isopropanol solution (3:2) was added and degreased by stirring. Then, after washing with ultrapure water, freeze-drying was performed to obtain a dried fish skin sample. A 100-fold amount of 0.2 M sodium hydroxide solution was added to 1 g of dried fish skin, and alkaline washing was performed by stirring for 24 hours. After washing with alkali, the mixture was washed with ultrapure water until it became neutral, and various enzymatic reaction solutions were added, followed by pressurization and heat treatment (2.0 kgf/cm^2, 121 ℃, 20 minutes) using an autoclave. After that, various enzymes were added to carry out enzymatic hydrolysis.

Table 1. shows the enzymes and decomposition conditions used. Alcalase (Sigma-Aldrich), papain, pepsin, trypsin (FUJIFILM Wako Pure Chemical Corp.), protease P "Amano" 3SD (Amano enzyme Inc.), and α-chymotrypsin (Tokyo Chemical Industry Co., Ltd.) were used for digestion (Nongonierma *et al*., 2018; Takahashi *et al*., 2021; Zhang *et al*., 2016). After enzymatic hydrolysis, the autoclave was again pressurized and heated to inactivate the enzyme, followed by centrifugation (14,010 g, 20 minutes), and the obtained supernatant was used as an enzymatic hydrolysis sample.

In addition, in order to recover low-molecular-weight components in the sample, ethanol was added to the yellowtail skin hydrolysate to a concentration of 75% [v/v], stirred, and then centrifuged (14,010 g, 20 minutes). Thereafter, ethanol was removed from the supernatant using a rotary evaporator to obtain an ethanol fraction.

Preparation of ethanol fraction from various fish skin hydrolysates

To compare the DPP-IV inhibitory activity of skin hydrolysates by fish species at the same concentration and clarify the difference, Alcalase 0.5% was used for fish skins of yellowtail, eel, flounder, mackerel, rainbow trout, and opah. Before adding the enzyme, the fish skins were treated in the same manner as in the previous enzymatic hydrolysis method. Enzymatic degradation was performed at 40 ℃ for 20 hours. Subsequently, ethanol fractionation was performed, and the DPP-IV inhibitory activity and weight average molecular weight of the supernatant were compared before and after ethanol fractionation and between fish species.

Artificial digestion test

The decomposition product of yellowtail skin obtained using 0.5% Alcalase was fractionated with ethanol and used for the artificial digestion test. After adjusting the ethanol fraction to pH 2.0 with hydrochloric acid, 1% (w/w) of porcine pepsin (Sigma-Aldrich) was added and reacted at 37 ℃ for 2 hours. After the reaction, the pH was adjusted to 8.0 with a sodium hydroxide solution, and the enzyme was deactivated by heating at 100 ℃ for 10 minutes. The pepsin digest was centrifuged (14,010 g, 20 minutes) to obtain a supernatant. Furthermore, 1% (w/w) of porcine trypsin (FUJIFILM Wako Pure Chemical Corp.) was added to the pepsin hydrolysate and reacted at 37 ℃ for 2 hours. After the reaction, the pepsin-trypsin hydrolysate was obtained by inactivating the enzyme by heating again. Each artificial digest obtained was centrifuged (14,010 g, 20 minutes), and the DPP-IV inhibitory activity and weight average molecular weight of the supernatant were examined.

Assay for DPP-IV inhibitory activity

To examine the DPP-IV inhibitory activity of various peptides, the method of Lacroix *et al*. (2012 and 2013) was partially modified. Using a 96-well microplate, 25 µl of each sample and 25 µl of 1.6 mM Gly-Pro-p-nitroanilide (final concentration 0.4 mM) were added and pre-incubated at 37 ℃ for 10 minutes in a plate incubator. Next, 50 µl of 4 mU/ml DPP-IV (final concentration 2 mU/ml) was added, stirred, and incubated at 37 ℃ for 1

Table 1. DPP-IV inhibitory activity, average molecular weight, and IC_{50} value of yellowtail skin hydrolysate before and after ethanol fractionation.

Enzyme (Conditions)	Hydrolysate		Ethanol fractionated		
	Specific activity (%/mg)	Molecular weight	Specific activity (%/mg)	Molecular weight	IC_{50} (mg/ml)
Alcalase (2%, 50 °C, pH 8.0)	342±2[a]	600±0	420±4[a*]	600±0	1.19±0.01[a]
Trypsin (1%, 37 °C, pH 8.0)	185±5[b]	9500±200	128±3[b*]	8900±100	3.90±0.08[b]
α-Chymotrypsin (4%, 37 °C, pH 8.0)	201±16[c]	10500±300	225±2[c]	6700±200[*]	2.22±0.02[c]
Protease P "Amano" 3SD (0.25%, 40 °C, pH 8.0)	297±9[d]	8900±0	331±4[d]	7700±100[*]	1.51±0.02[d]
Control (40 °C, pH 8.0)	23±2	-	-	-	-
Papain (3%, 50 °C, pH 7.0)	249±11[e]	9100±100	184±5[e*]	8300±200	2.72±0.07[e]
Control (50 °C, pH 7.0)	60±14	-	-	-	-
Pepsin (3%, 37 °C, pH 2.0)	168±19[f]	8800±100	112±3[f]	7100±100[*]	4.46±0.11[f]
Control (37 °C, pH 2.0)	60±16	-	-	-	-

All data are shown as the mean ± SE (n=3). The different letters indicate significant differences by Tukey-Kramer ($p < 0.05$). The significant difference in specific activity and molecular weight before and after ethanol fractionation are indicated by the asterisk by Student's *t*-test ($p < 0.05$).

hour. After completion of the reaction, 1 M sodium acetate buffer (pH 4.0) was added to stop the reaction, and absorbance (405 nm) was measured using a microplate reader (SH9000, Hitachi). Diprotin A (PEPTIDE INSTITUTE, Inc.), an inhibitor, was also measured simultaneously (Lacroix *et al*., 2013). DPP-IV inhibitory activity was calculated as follows.

$$\text{DPP-IV Inhibition (\%)} = 1 - \left(\frac{\text{Sample} - \text{Sample control}}{\text{Negative reaction} - \text{Negative control}}\right)$$

Sample: with inhibitor and DPP-IV.
Sample control: with inhibitor and without DPP-IV.
Negative reaction: without inhibitor and with DPP-IV.
Negative control: without DPP-IV and inhibitor.

IC_{50} values were determined to compare the inhibitory effects of various degradation products. Furthermore, to examine the action on human DPP-IV, the DPP-IV Drug Discovery kit (Enzo Life Sciences, Inc.) and Diprotin A were used to compare IC_{50}.

To investigate the DPP-IV inhibitory properties of degradation products obtained from yellowtail skins, Lineweaver-Burk plots were used to examine the inhibitory mode of peptides against DPP-IV (final concentration 2 mU/ml) (Umezawa *et al*., 1984).

Weight average molecular weight and amino acid composition analysis

The weight average molecular weight of fish skin hydrolysate was analyzed by size exclusion column chromatography (SEC). Various degradants dissolved in HPLC eluent, 0.1% [v/v] trifluoroacetic acid–30% acetonitrile, were used for SEC.

The HPLC analysis conditions were Shimadzu LC-20 system, Superdex™ peptide 10/300GL (Cytiva), column temperature of 40 °C, flow rate of 0.5 ml/min, and absorption wavelength of 230 nm. Lysozyme and L-anserine from Sigma-Aldrich and insulin, substance P, and glycylglycine from FUJIFILM Wako Pure Chemical

Corp were used as standard substances. The lab solution GPC Software (Shimadzu Corporation) was used as SEC analysis software, and a calibration curve of standard substances was created to calculate the weight average molecular weight.

Amino acid composition analysis was performed on yellowtail skin and ethanol fractions using the post-column with the o-phthalaldehyde (OPA) reagent (Saito *et al*., 2014). In addition, acid-soluble type I collagen (ASC) was prepared from the skin of the yellowtail, and the amino acid composition was analyzed in the same manner.

Statistical analysis

Analysis results such as protein concentration and DPP-IV inhibitory activity are shown as mean ± standard error using data obtained from three or more measurements. Statistical analysis software SPSS Statistics ver. 28 (IBM) was used to perform one-way analysis of variance, Tukey-Kramer method, and Student's t-test. In both cases, the significance level was set at 5%.

RESULT

Various enzymatic degradation

DPP-IV inhibitory activity was observed in all enzymatic hydrolysates (Table 1). In addition, the inhibitory activity against decomposition products of Alcalase, 3SD, and papain was high. Next, when the hydrolysate was fractionated with ethanol, the average molecular weight decreased in the hydrolysate other than in Alcalase. The IC_{50} of those fractions was the lowest for Alcalase, showing 1.19 mg/ml.

Changes in Alcalase decomposition products at different reaction temperatures

In order to efficiently obtain enzymatic decomposition products, the amount of Alcalase, temperature, and reaction time were investigated using dried yellowtail skin after alkali washing. When enzymatic degradation was performed at reaction temperatures of 50 °C and 40 °C, the specific activity for DPP-IV inhibition was the highest at 20 hours of degradation conditions (Table 2). At both 40 °C and 50 °C, the weight-average molecular weight of the degradation products was about 1200 at 2% enzyme. On the other hand, in the case of 0.5% enzyme at 40 °C, the weight average molecular weight was 2300.

As a result of enzymatic hydrolysis of various fish skins under the same conditions, yellowtail skin hydrolysate had the highest DPP-IV inhibitory activity, followed by eel (Figure 1). In addition, the specific activity increased significantly after fractionation compared to before ethanol fractionation.

Amino acid composition of yellowtail ethanol fraction

The amino acid composition of enzymatic hydrolysate of yellowtail with low IC_{50} was investigated and compared with untreated fish skin before decomposition and ASC (Table 3). Gly was the most abundant in the decomposed product and untreated fish skin, followed by Pro, Hyp, Ala, etc., showing a composition similar to ASC. Comparing the decomposed product with undegraded yellowtail skin, the decomposed product had significantly higher in Pro, Met and Tyr ratios and lower in Leu. Hydrolysis with Alcalase confirmed differences in amino acid residues contained in the recovered hydrolysates.

Lineweaver-Burk plot for DPP-IV

The inhibitory mode against DPP-IV was investigated in ethanol fractions of yellowtail after Alcalase digestion (Figure 2.). Diprotin A, which was used as a positive control, had an IC_{50} of 5.2 µM and a high Km (3.35 mM), and did not affect Vmax (0.013), confirming competitive inhibition against DPP-IV. In contrast, the ethanol fraction of the yellowtail after Alcalase digestion showed a similar effect to the mixed inhibition type as there was a slight increase in Km (1.50 mM) and a slight decrease in Vmax (0.007).

Human DPP-IV inhibitory activity and artificial digestion test

To compare the effects of DPP-IV in different mammalian species, porcine and human DPP-IV were used to determine the IC_{50} values of Diprotin A and ethanol fractions of yellowtail. Diprotin A had an IC_{50} of 5.2±0.02 µM and 5.6 µM ± 0.05 µM against porcine and human DPP-IV, respectively. Ethanol fractions of yellowtail also showed similar IC_{50} values (data not shown), which were not significantly different.

As a result of the artificial digestion test with pepsin and pepsin-trypsin of yellowtail ethanol fraction, no change in activity and molecular weight was observed in the pepsin-digested product and the pepsin-trypsin digested product compared to the undigested hydrolysate (Figure 3.).

DISCUSSION

In this study, as a result of degrading using 6 types of enzymes, differences in DPP-IV inhibitory activity and weight average molecular weight were observed depending on the enzyme. These are due to differences in

Table 2. DPP-IV inhibitory activity of Alcalase hydrolysate from yellowtail skin at different temperatures and degradation times.

Temperature (Enzyme amount)	Degradation time	Specific activity (%/mg)	Total activity (%/total protein amounts)	Average molecular weight
40 °C (0.5%)	1h	259 ± 9^{aA}	9700 ± 300^{aA}	-
	3h	281 ± 4^{abA}	10500 ± 200^{abA}	-
	6h	297 ± 1^{bcA}	11200 ± 100^{bA}	-
	20h	317 ± 4^{cA}	10900 ± 100^{bA}	2300 ± 100^{a}
40 °C (2%)	1h	309 ± 6^{aB}	12000 ± 100^{aB}	-
	3h	331 ± 7^{abB}	12200 ± 100^{aB}	-
	6h	321 ± 7^{abAB}	10800 ± 300^{aA}	-
	20h	349 ± 7^{bA}	12700 ± 600^{aA}	1200 ± 0^{b}
50 °C (2%)	1h	317 ± 10^{aB}	12000 ± 300^{aB}	-
	3h	321 ± 6^{aB}	11600 ± 200^{abB}	-
	6h	339 ± 9^{aB}	10700 ± 300^{abA}	-
	20h	325 ± 5^{aA}	10400 ± 100^{bA}	1200 ± 0^{b}

All data are presented as mean ± SE (n=3). Significant differences at the same temperature and enzyme amount are shown in different lowercase letters and significant differences at the same time are shown in different uppercase letters, all in Tukey Kramer ($p < 0.05$).

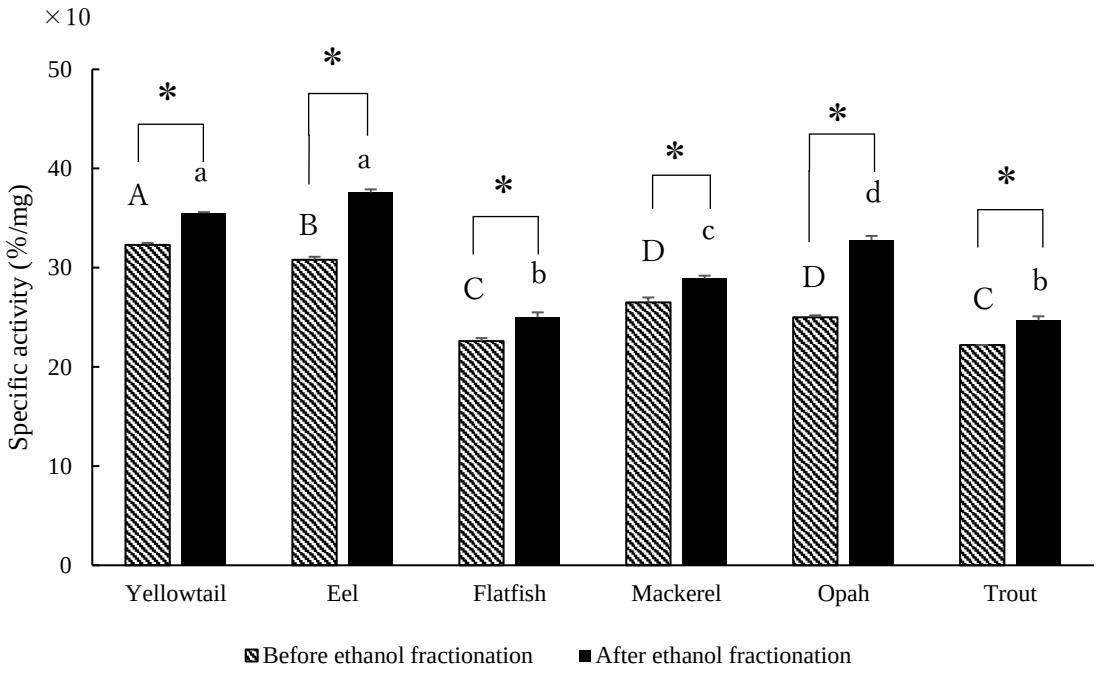


Figure 1. DPP-IV specific activity of fish skin hydrolysate before and after ethanol fractionation. Bars represent standard errors from triplicate determinations. Different letters indicate significant difference between samples by Tukey-Kramer ($p<0.05$). The significant difference in specific activity before and after ethanol fractions are indicated by asterisks by Student's *t*-test ($p<0.05$).

the substrate specificity of the enzymes. Alcalase hydrolysate, which showed high inhibitory activity in degradation products, has been reported to recognize amino acid residues such as aromatic (Phe, Trp, Tyr), acidic (Glu), basic (Lys), Sulfur-containing (Met), aliphatic (Leu and Ala), and hydroxyl group (Ser) (Doucet *et al*., 2003). Therefore, it is thought that the production of many peptides with various fragments affected the DPP-IV inhibitory activity. Alcalase hydrolysate showed a weight average molecular weight of 600. It is widely known that molecular weight distribution may affect the physiological activity of protein hydrolysates, and peptides with a molecular weight of less than 1000 are thought to play a major role in DPP-IV inhibitory activity (Agustia *et al*., 2023).

Alcalase is an endoprotease with broad specificity and is inexpensive, so it is used for industrial production, such as meat tenderization, cheese flavoring, baked confectionery production, and animal feed digestibility improvement (Güntelberg *et al*., 1952; Smith *et al*., 1968; Kumar *et al*., 1999). For the industrial production of peptides, cost reduction and production efficiency are major issues. After examining the degradation reaction time, temperature, and the amount of enzyme in Alcalase, it was found that the sample with 2% Alcalase increased DPP-IV inhibitory activity regardless of temperature. On the other hand, even if the degradation time was increased to 20 hours, no significant change was observed in the difference in inhibitory activity. Next, when the enzyme amount was reduced to 0.5%, the inhibitory activity of these hydrolysates was low in a short period of 1 to 6 hours. However, when hydrolyzed for 20 hours, the inhibitory activity was high. It exhibited almost the same activity as the degradation product with an enzyme amount of 2%. From the above, it is considered that the enzyme amount of 0.5% and the long decomposition time are suitable as enzymatic decomposition conditions.

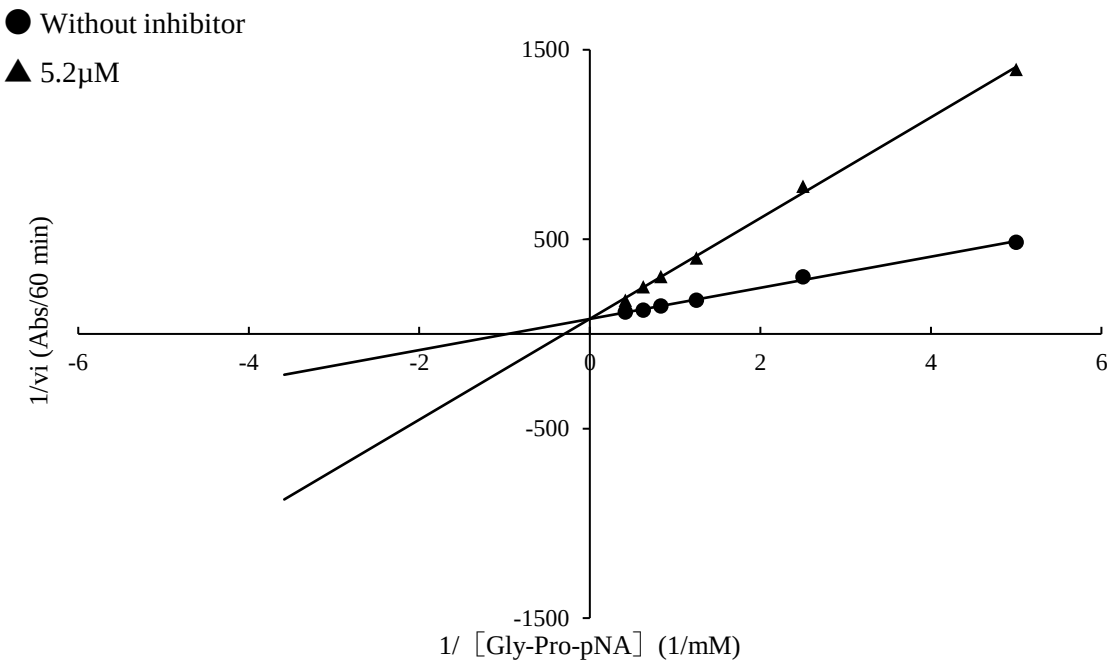
The yellowtail hydrolysate obtained by six kinds of enzymes was further fractionated with ethanol, and the As eight-average molecular weight was measured. a result,

Table 3. Amino acid composition of yellowtail hydrolysate after ethanol fractionation (%)

	Hydrolysate	Untreated skin	ASC
Asp	4.6±0.0^{ab}	5.0±0.2^{a}	4.5±0.0^{b}
Hyp	6.4±0.1^{a}	5.6±0.3^{a}	6.7±0.2^{a}
Thr	2.6±0.0^{a}	2.8±0.5^{a}	2.5±0.0^{a}
Ser	3.8±0.1^{a}	3.9±1.1^{a}	3.7±0.1^{a}
Glu	8.0±0.1^{a}	8.2±0.5^{a}	8.1±0.2^{a}
Pro	11.4±0.1^{a}	9.3±0.6^{b}	11.0±0.2^{a}
Gly	32.8±0.2^{a}	33.9±1.6^{a}	33.8±0.8^{a}
Ala	12.4±0.1^{a}	12.6±0.8^{a}	12.0±0.1^{a}
Val	2.3±0.0^{a}	2.3±0.4^{a}	1.8±0.3^{a}
Met	0.9±0.1^{a}	0.0±0.0^{b}	1.2±0.0^{c}
Ile	1.4±0.0^{a}	1.5±0.2^{a}	1.2±0.1^{a}
Leu	2.4±0.0^{a}	2.9±0.2^{b}	1.9±0.1^{c}
Tyr	0.4±0.0^{a}	0.0±0.0^{b}	0.4±0.1^{c}
Phe	1.5±0.0^{a}	1.7±0.2^{a}	1.4±0.0^{a}
His	0.9±0.1^{a}	0.8±0.1^{a}	0.6±0.0^{b}
Hyl	0.4±0.0^{a}	0.5±0.1^{a}	0.6±0.0^{b}
Lys	2.7±0.0^{a}	3.2±0.5^{a}	3.1±0.2^{a}
Arg	5.0±0.1^{a}	5.6±0.6^{a}	5.6±0.2^{a}
Total	100	100	100

The different letters indicate significant differences by Tukey-Kramer ($p < 0.05$). ASC means acid-soluble type I collagen prepared from yellowtail skin. All data are shown as the mean ± SE (n=3).

(A) Diprotin A



(B) Yellowtail after ethanol fractionation

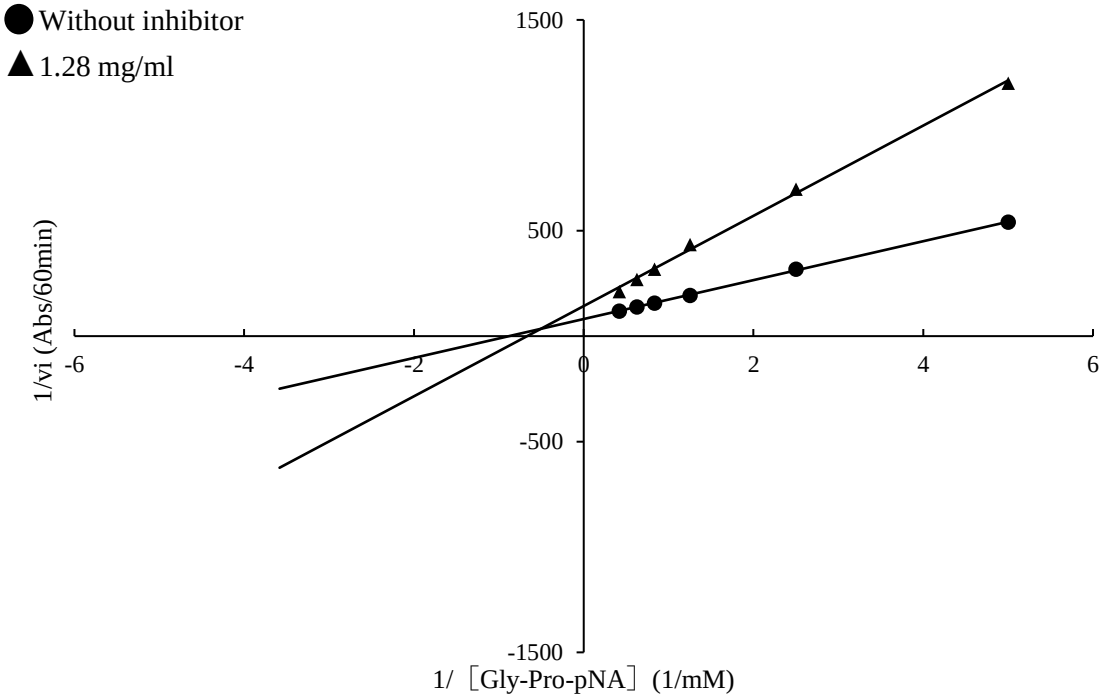


Figure 2. Lineweaver-Burk plot for DPP-IV inhibition with diprotin A (A) and yellowtail hydrolysate after ethanol fractionation. In the assay, the hydrolysate was measured at concentrations close to the IC_{50}.

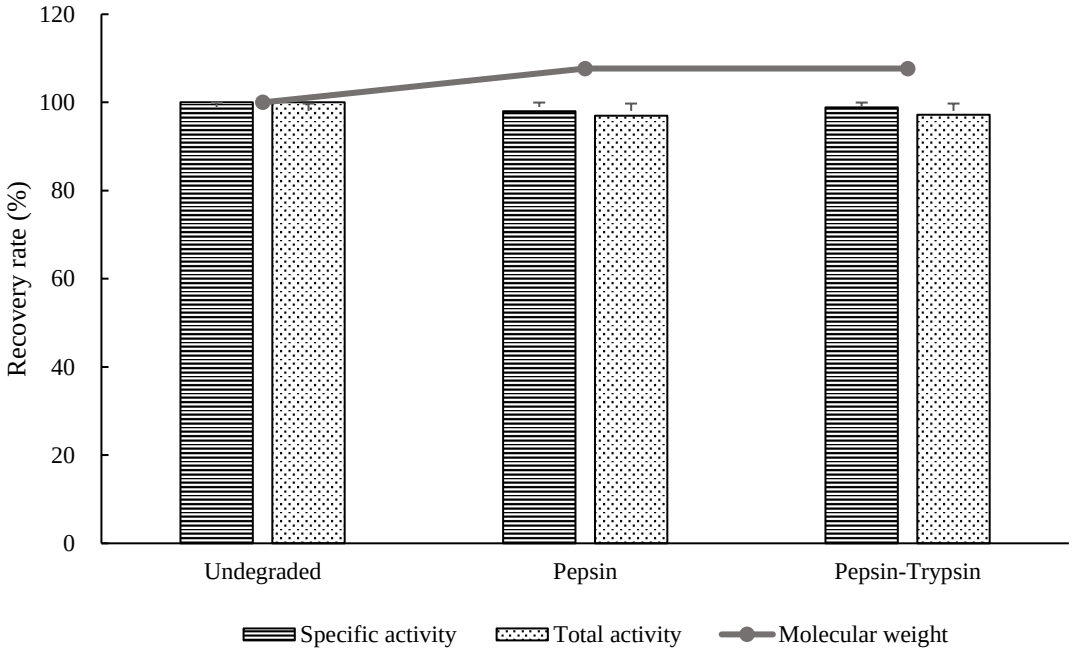


Figure 3. Recovery rate of ethanol fraction of yellowtail hydrolysate after simulated gastrointestinal digestion. Bars represent standard errors from triplicate determinations.

there was no difference in the molecular weight of Alcalase degradation products before and after fractionation. On the other hand, the molecular weight decreased in all other enzymatic degradation products.

The IC_{50} measured after ethanol fractionation was significantly lower for the Alcalase hydrolysate. These results suggest that the DPP-IV inhibitory peptides in the degradation products may be related to their molecular weight and hydrophobicity.

Amino acid composition analysis of the ethanol fraction of yellowtail, which had high DPP-IV inhibitory activity, revealed that following Gly, imino acids (Pro and Hyp), and Ala were the highest. It was presumed that the resulting degradation products were peptides derived from type I collagen, a major component of fish dermis (Saito *et al*., 2014). Comparing the undegraded yellowtail skin with the decomposed product, the Pro content of the decomposed product was significantly higher. It is presumed that the enzymatic hydrolysis of fish skin partially purified a peptide containing many Pro residues, which directly affects the inhibition of DPP-IV (Nongonierma *et al*., 2013).

Currently reported IC_{50} (mg/ml) of collagen peptide that inhibits DPP-IV is 1.65 to 3.04 for bovine skin, 1.5 to 1.61 for porcine skin, 3.52 for chicken leg, and 1.35 for salmon skin (Hatanaka *et al*., 2014; He *et al*., 2023; Hsu *et al*., 2013; Li-Chan *et al*., 2012). In this study, the IC_{50} values of the yellowtail and eel skin ethanol fractions after Alcalase digestion obtained are lower than those previously reported, suggesting that their inhibitory activity against DPP-IV is high.

DPP-IV inhibitory peptides have been reported to exhibit various inhibition modes, such as competitive, non-competitive, uncompetitive, and mixed types, and bind to the active site and other sites in the structure of DPP-IV (Liu *et al*., 2013). Food-derived peptides such as camel milk protein and soft-shelled turtle egg yolk have been reported to show competitive inhibition (Nongonierma *et al*., 2018; Nong *et al*., 2013). Diprotin A, used as a control, showed competitive inhibition, and the ethanol fraction of yellowtail skin after Alcalase digestion showed mixed inhibition with competitive and non-competitive properties. These results suggested that the degradation products contained peptides with various inhibitory modes.

To compare the effect of pig-derived DPP-IV and human-derived DPP-IV on activity, we compared the IC_{50} of the ethanol fraction of yellowtail after digestion with Alcalase, along with diprotin A as a positive control. As a result, there was no significant difference in inhibitory activity between human and porcine DPP-IV. Furthermore, in order for DPP-IV inhibitory peptides to be ingested by humans and show efficacy, they must not be degraded in the digestive system. After conducting an

artificial digestion test, no significant changes were observed in DPP-IV inhibitory activity or weight average molecular weight, indicating no effect on the human digestive absorption process. When humans ingest these peptides, similar to *in vitro* results, they inhibit DPP-IV present in the human body and inhibit the degradation of incretins, possibly contributing to improved blood sugar control.

Based on the above, the DPP-IV inhibitory effect of fish skin hydrolysate was clarified in this study, and the basis for efficient peptide production was established by examining enzymatic decomposition conditions and fractionation methods. As the raw material is normally discarded skin, production costs can be kept low, and the ethanol fraction after enzymatic degradation has the advantage that peptides with enhanced DPP-IV inhibitory activity can be recovered. Therefore, DPP-IV inhibitory peptides obtained from fish skin may be applied as food ingredients in various foods such as soups, beverages, health foods, and supplements in the future.

CONCLUSION

In this study, the skins of yellowtail, eel, flounder, mackerel, rainbow trout, and opah were used to prepare hydrolysates that exhibit DPP-IV inhibitory activity. After pressurization and heating, hydrolysis with several kinds of enzymes showed high DPP-IV inhibitory activity with Alcalase. Alcalase digestion of each fish skin revealed that yellowtail and eel had the lowest IC_{50} values, suggesting that the low-molecular-weight fraction contained peptides that inhibit DPP-IV. As a result of the artificial digestion test, there was no difference in the weight average molecular weight and DPP-IV inhibitory activity before and after the digestion test, suggesting that it is difficult to be degraded by digestive enzymes when ingested by humans. In the future, it is necessary to confirm the efficacy and safety *in vivo* and to investigate the structure and content of specific components that exhibit inhibitory activity.

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