



# Optimisation of Protease Hydrolysis Conditions for ACE-Inhibitory Peptide Extraction From Sandbar Shark (*Carcharhinus plumbeus*) Muscle

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## Abstract

Given the abundance and cost-effectiveness of catching sandbar sharks (*Carcharhinus plumbeus*), their muscle protein may serve as a valuable alternative protein source, particularly for the elderly, while also promoting the sustainable utilisation of marine resources. Food-processing protease is an effective tool to improve the texture and taste of food, enhancing intake in elderly people. The utilisation of sandbar shark muscle as a functional food ingredient was investigated through protease hydrolysis and the identification of angiotensin-converting enzyme (ACE) inhibitory peptides. Among various protease treatments, Papain W-40, Protin SD-AC10F, and Thermoase PC10F hydrolysis exhibited stronger inhibition. By comparing protease reaction times and  $IC_{50}$  values, we determined the optimal condition to be Thermoase PC10F for 12 h, which yielded the highest ACE inhibitory activity with minimal bitterness. Fractionation of the hydrolysate revealed multiple potential active peptides, with the most potent fraction containing an active peptide identified as Ala-Phe. The synthesised Ala-Phe exhibited an  $IC_{50}$  of  $93.8 \mu\text{g}\cdot\text{mL}^{-1}$  and demonstrated a non-competitive inhibition mechanism against ACE. This study highlights the potential of sandbar shark muscle hydrolysates as a novel functional food ingredient with antihypertensive properties.

**Keywords:** hydrolysate, peptide, functional food

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## Introduction

As the global population continues to age, sarcopenia – the progressive loss of muscle mass and function – has emerged as a critical public health issue (Cruz-Jentoft & Sayer, 2019). This condition not only impairs physical performance but also increases the risk of immobility and prolonged bedridden states, ultimately leading to a decline in overall quality of life. To mitigate the progression of sarcopenia, adequate protein intake is essential, with particular emphasis on high-quality animal proteins that offer a well-balanced amino acid composition (Coelho-Junior et al., 2022).

Fish meat is recognised as a nutritionally valuable protein source due to its high protein content and low-fat levels, setting it apart from terrestrial livestock meat. Given these attributes, fish-derived proteins

have garnered attention for their health benefits, leading to the development of various functional food products utilising fish protein.

The potential of shark meat as a protein source has gained increasing attention (Chen, 1995; Mathew et al., 2002). The coastal waters of Japan are home to a diverse range of shark species, broadly classified into deep-sea and shallow-water species. Deep-sea sharks are commercially exploited for their livers, which contain squalene, a valuable compound used in pharmaceuticals and cosmetics (Mendes et al., 2022). In contrast, shallow-water sharks, which lack squalene in their livers, are not typically targeted for commercial fishing. In Kagoshima Prefecture, located in the southern part of Kyushu Island in Japan, large shallow-water sharks pose a significant challenge to the local fishing industry, as they prey on fish caught in longline fisheries and pose risks to fishers.

Despite their limited commercial use, large sharks serve as a rich source of high-quality myofibrillar proteins, and their substantial muscle mass presents a significant advantage for food production. However, one drawback of shark meat is its tendency to generate ammonia as freshness declines, which can impact its sensory properties. Nevertheless, when consumed in its fresh state, shark meat remains a viable and nutritious protein source.

One of the challenges in utilising animal meat for human consumption, particularly among elderly individuals, is reduced masticatory function, which makes it difficult to consume certain protein-rich foods. While mincing can improve ease of swallowing, it may also negatively affect the food's appearance, potentially leading to a decrease in appetite. To address these concerns, food-processing proteases have been employed to modify the texture and flavour of protein sources. Enzymatic treatment can enhance the tenderness of shark meat while preserving its nutritional value, making it a promising dietary protein source for an ageing population.

In addition to improving texture, protease treatment results in the breakdown of proteins into smaller peptides, some of which may exhibit bioactive properties. Among age-related health concerns, hypertension is particularly prevalent in elderly populations and significantly increases the risk of cardiovascular and cerebrovascular diseases. These conditions not only pose severe health risks but also contribute to long-term immobility and bedridden states.

Given the need for both muscle preservation and cardiovascular health in ageing individuals, the valorisation of by-products generated during shark meat processing could offer a dual benefit: aiding in sarcopenia prevention while providing bioactive peptides with potential health-promoting properties. Since hypertension is closely linked to the activity of angiotensin-converting enzyme (ACE), which catalyses the conversion of angiotensin I to angiotensin II, inhibiting ACE is a well-recognised strategy for managing hypertension in ageing population. The sandbar shark (*Carcharhinus plumbeus*) is widely distributed in tropical and subtropical regions of the Pacific Ocean, the Indian Ocean, the Atlantic Ocean, and the Mediterranean, and can grow to over 2 m in length (Joung et al., 2004). Therefore, this study aims to evaluate the ACE inhibitory effects of peptides derived from protease-treated sandbar shark meat, exploring their potential as functional food components for improving elderly health.

## Materials and Methods

### Ethical approval

Minced muscle tissue of the sandbar shark (*Carcharhinus plumbeus*) was obtained from the Kagoshima Prefectural Fisheries Technology and

Developmental Center, Ibusuki, Japan. No live animals were used, handled, or sacrificed in this study. Consequently, institutional animal care and use committee (IACUC) approval was not required for this investigation.

### Materials

Minced muscle of sandbar shark was kindly provided by Kagoshima Prefectural Fisheries Technology and Developmental Center, Ibusuki, Japan. The sample was kept at  $-80^{\circ}\text{C}$  until use. Food-processing enzymes were obtained from AMANO Enzyme (Nagoya, Japan). Ala-Phe was commercially synthesised by SCRUM Inc. (Tokyo, Japan).

### Preparation of shark muscle hydrolysates

Minced raw sandbar shark muscle was boiled in water to inactivate endogenous proteases and then centrifuged at 12,000 g for 30 min. One gram of the precipitate was digested with 7.5 mg of food-processing enzymes, including Umamizyme (Uma, 70 unit.g<sup>-1</sup>), Newlase F3G (Ne, 7,000 unit.g<sup>-1</sup>), Peptidase R (Pep, 420 unit.g<sup>-1</sup>), Papain W-40 (Pa, 400 unit.g<sup>-1</sup>), Protease A "Amano" SD (ProA, 10,000 unit.g<sup>-1</sup>), Protease M "Amano" SD (ProM, 5,500 unit.g<sup>-1</sup>), Protease P "Amano" 3SD (ProP, 30,000 unit.g<sup>-1</sup>), Protin SD-AC10F (Pr, 90,000 PU.g<sup>-1</sup>), Thermoase PC10F (Th, 90,000 PU.g<sup>-1</sup>), or Porcine Pepsin (Wako Chemicals, Osaka, Japan), in 5 mL of water adjusted to the optimal pH for 5 h. Afterward, the mixture was boiled again to inactivate the food-processing enzymes. After centrifugation at 12,000 g for 30 min, the obtained supernatants were used as the muscle hydrolysates for the ACE inhibitory assay.

### Bitterness evaluation of hydrolysates

The bitterness of hydrolysates was evaluated according to our previous study (Ikeda et al., 2015). A taste panel consisting of ten individuals evaluated the bitterness intensity, which was categorised as follows: "±" (no bitterness), "+" (slight bitterness), "++" (moderate bitterness), and "+++" (strong bitterness). The sensory panel was not formally trained; therefore, the bitterness evaluation should be interpreted as semiquantitative.

### Evaluation of ACE inhibitory activity

The ACE inhibitory activity was determined using the previously described method with slight modifications (Shiozaki et al., 2010). A sample solution (15  $\mu\text{L}$ , equivalent to 3 mg of shark muscle) was mixed with 125  $\mu\text{L}$  of 7.6 mM hippuryl-histidyl-leucine (Hip-His-Leu) in 100 mM borate buffer (pH 8.3) containing 200 mM NaCl and incubated with 50  $\mu\text{L}$  of ACE (3 mU) at  $37^{\circ}\text{C}$  for 1 h. The sample was applied to a cholesterol-binding column (COSMOSIL Cholester, 4.6 mm i.d.  $\times$  150 mm, Nacalai Tesque, Tokyo, Japan).

The column was eluted with a solvent mixture of 10 mM KH<sub>2</sub>PO<sub>4</sub> (pH 3.0) and methanol (1:1) at a flow rate of 1 mL·min<sup>-1</sup>. The eluate was monitored at 230 nm, and the amount of hippuric acid released by ACE was quantified.

The inhibition rate was calculated using the following formula: ACE inhibitory activity (%) = [(Ec - Es) / (Ec - Eb)] × 100, where Es represents the amount of hippuric acid released in the presence of the test sample, Ec represents the amount of hippuric acid released in the absence of the test sample, and Eb represents the amount of hippuric acid released in the absence of ACE. The IC<sub>50</sub> value was defined as the concentration required to achieve 50 % ACE inhibition.

### Identification of ACE inhibitory peptide

Shark muscle hydrolysate was subjected to dialysis using a membrane with a molecular weight cut-off (MWCO) of 14,000 Da. The low-molecular-weight fraction (<14,000 Da) was analysed using a high-performance liquid chromatography (HPLC) system consisting of two pumps (LC-10ATvp, SHIMADZU, Kyoto, Japan), a photodiode array detector (SPD-M10Avp, SHIMADZU), a system controller (SCL-10Avp, SHIMADZU), and a data analyser (CLASS-vp, SHIMADZU). Separation was performed on a COSMOSIL 5C18-MS column (10 mm i.d. × 250 mm, Nacalai Tesque) using a linear gradient from 0.1 % trifluoroacetic acid to 0.1 % trifluoroacetic acid in 90 % methanol at a flow rate of 3 mL·min<sup>-1</sup> for 30 min. Elution was monitored at 250 nm. Individual fractions were lyophilised, dissolved in water, and assessed for ACE inhibitory activity. The active fraction was further purified using a COSMOSIL 5C18-MS II column (4.6 mm i.d. × 250 mm, Nacalai Tesque) with a methanol gradient. Amino acid composition was analysed using amino acid analysis and an amino acid sequencer (Ikeda et al., 2015).

### Determination of the ACE inhibition pattern

A kinetic study was performed under identical conditions with varying concentrations of Hip-His-Leu (0.53–2 mM). The experiments were conducted using peptide concentrations of 0, 100, 200, and 400 nmol·mL<sup>-1</sup>. The inhibition patterns of ACE were determined using Lineweaver-Burk plots.

## Statistical analysis

All data were expressed as means ± standard deviations (SD). Statistical significance among enzyme treatments was analysed using one-way ANOVA followed by Tukey's post-hoc test. A significance threshold of *P* < 0.05 was applied. Significant differences are indicated by different letters in Figures 1–2.

## Results

### Optimal conditions for the preparation of hydrolysate from sandbar shark muscle using food-processing proteases

Proteolytic digestion of sandbar shark muscle was performed by nine different food-grade proteases and one research-grade protease for 5 h. The yield of proteolytic hydrolysates per gram of shark muscle varied depending on the protease used, with values of 13.75 (Uma), 11.25 (Ne), 9.1 (Pa), 8.15 (Pep), 13.05 (ProA), 11.85 (ProM), 14.3 (ProP), 11.1 (Pr), 13.75 (Th), and 1.15 mg (pepsin). In the absence of protease treatment, the yield was 1.2 mg per gram of muscle.

For its application as a food ingredient, taste alterations are an important factor. Since some amino acids impart bitterness, we first examined whether protease treatment could induce bitterness (Table 1). Sensory evaluation revealed that the hydrolysates treated with Pa and Pr exhibited slight bitterness, whereas noticeable bitterness was observed in the hydrolysate obtained from ProP treatment. The remaining hydrolysates exhibited negligible bitterness.

Subsequently, the ACE inhibitory activity of the hydrolysates was assessed (Fig. 1). The inhibitory activity ranged from more than 80 % to less than 10 % in the hydrolysates, while no inhibition was observed in the non-hydrolysate treatment. Among the hydrolysates, Th exhibited the greatest inhibition, followed by Pap and Pr. To determine the optimal reaction time, hydrolysis was performed for 1, 5, and 12 h using Th, Pap, and Pr proteases (Fig. 2). The strongest inhibition in Pap and Pr treatments was observed after 5 h of digestion, whereas Th exhibited the highest inhibitory activity after 12 h. The bitterness of the 12 h hydrolysate of Thermoase PC10F was also evaluated and was found to be minimal, similar to that of the 5 h hydrolysates.

Table 1. Bitterness of sandbar shark muscle hydrolysate.

	Uma	Ne	Pa	PeP	ProA	ProM	ProP	Pr	Th
Bitterness	±	±	+	±	±	±	++	+	±

Bitterness scale: "±" (no bitterness), "+" (slight bitterness), "++" (moderate bitterness), and "+++" (strong bitterness).

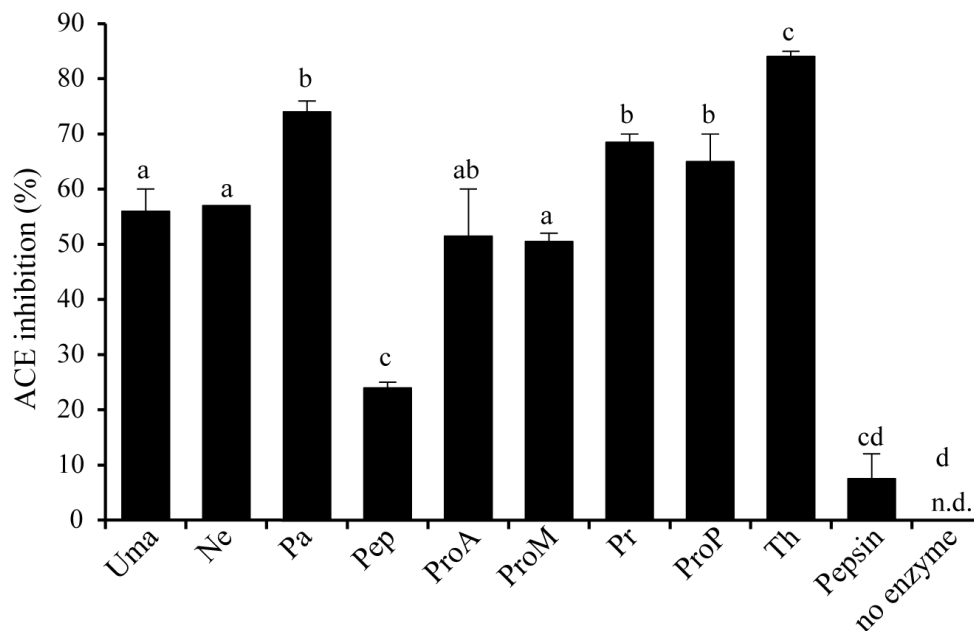


Fig. 1. Angiotensin-converting enzyme (ACE) inhibitory activity of the sandbar shark muscle hydrolysates. Ten shark muscle hydrolysates were evaluated for ACE inhibitory activity. The “No enzyme” sample refers to the prepared sample without food-processing enzymes. The enzymes used were Umamizyme (Uma), Newlase F3G (Ne), Peptidase R (Pep), Papain W-40 (Pa), Protease A “Amano” SD (ProA), Protease M “Amano” SD (ProM), Protease P “Amano” 3SD (ProP), Protin SD-AC10F (Pr), Thermoase PC10F (Th), or Porcine Pepsin. n.d., not detected.  $n = 5$ . Results are expressed as the mean  $\pm$  standard deviation. Columns having the same letter are not statistically different, and columns with different letters differed significantly ( $P < 0.05$ ).

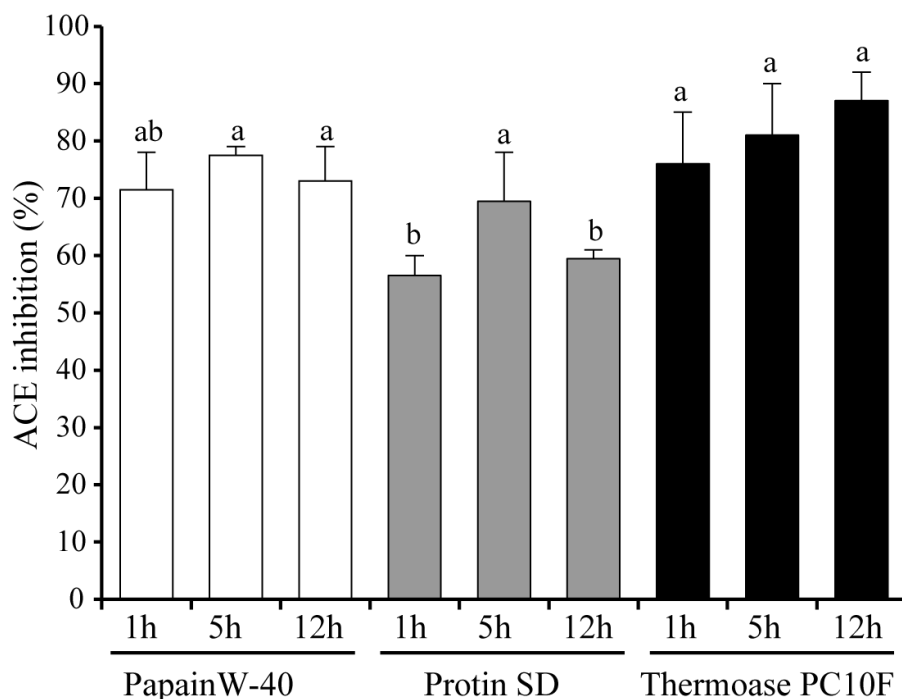


Fig. 2. Angiotensin-converting enzyme (ACE) inhibitory activity of the sandbar shark muscle hydrolysates prepared by Papain W-40, Protin SD, and Thermoase PC10F with different hydrolysis time. The ACE inhibitory activity of the hydrolysates was evaluated. Samples were prepared using Papain W-40, Protin SD, or Thermoase PC10F with different hydrolysis times (1, 5, and 12 h).  $n = 5$ . Results are expressed as the mean  $\pm$  standard deviation. Columns having the same letter were not statistically different, and columns with different letters differed significantly ( $P < 0.05$ ).

Subsequently, the  $IC_{50}$  values, representing the concentration required to inhibit 50 % of ACE activity, were determined for the 5 h hydrolysates of Pap and Pr, and the 12 h hydrolysate of Th (Fig. 3). The  $IC_{50}$  values were 85.4, 172.4, and 267.5  $\mu\text{g}\cdot\text{mL}^{-1}$  for Th, Pap, and Pr, respectively. Based on these results, the present study determined the optimal conditions for proteolytic hydrolysis of sandbar shark muscle as the 12 h treatment with Th protease that exhibited strong ACE inhibitory activity while maintaining low bitterness.

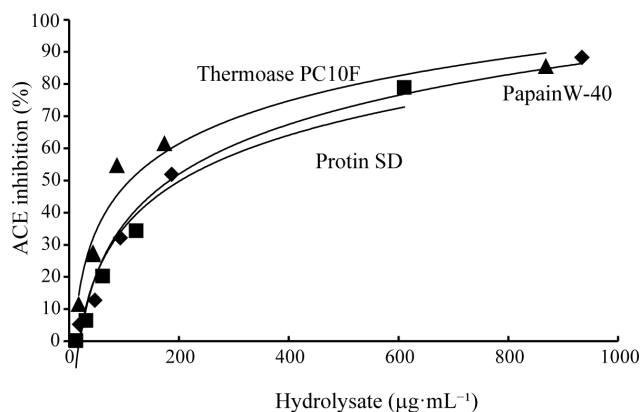


Fig. 3. Dose-dependent angiotensin-converting enzyme (ACE) inhibition by Thermoase, Papain and Protin hydrolysates of sandbar shark muscle. ACE inhibition by Thermoase (closed triangle), Papain (closed diamond), and Protin hydrolysate (closed square) was estimated with different doses in the reaction mixture.  $n = 5$ . The results are shown as the mean  $\pm$  standard deviation.

### Identification of the most active peptide showing ACE inhibitory activity in the shark muscle hydrolysate

Based on these findings, the optimal enzymatic treatment condition for sandbar shark muscle was determined to be a 12-h treatment with Thermoase PC10F; thereafter, we proceeded to identify the most effective peptide within this hydrolysate. The fraction obtained by dialysis of the hydrolysate with a molecular weight cut-off of 14,000 Da was then analysed for ACE inhibitory activity. The ACE inhibitory activity was 38.2 % and 50.9 % in the low- and high-molecular weight fractions, respectively. Since long-chain peptides are generally not efficiently absorbed in the intestine, we focused on the low-molecular-weight fraction. This low-molecular-weight fraction was separated using HPLC, which yielded ten fractions (Fr.1–Fr.10; Fig. 4A). Comparison of their ACE inhibitory activities revealed that all fractions exhibited high ACE inhibition, indicating that the ACE inhibition of sandbar shark muscle hydrolysate was attributed to multiple peptides. Among them, Fr.5 exhibited the highest ACE inhibition, prompting further fractionation. Fr.5 was separated into four subfractions (Fr.5A, Fr.5B, Fr.5C, and Fr.5D), which were evaluated for their ACE

inhibitory activities (Fig. 4B). The results demonstrated that Fr.5C exhibited the strongest ACE inhibitory activity among the fractions. Fr.5C displayed a single peak and was purified next for amino acid sequence determination using an amino acid sequencer (Fig. 4C). The peptide was identified as Ala-Phe. To confirm its identity, synthetic Ala-Phe was synthesised and its retention time was compared with that of the isolated Fr.5C, showing a 100 % match (Fig. 4D). Based on comparison with the synthesised peptide standard, 0.57 mg of Ala-Phe was obtained from 1 g of raw muscle treated with Thermoase PC10F. The  $IC_{50}$  value of the synthesised Ala-Phe was found to be 93.8  $\mu\text{g}\cdot\text{mL}^{-1}$  (Fig. 4E). Based on the secondary plot of the y-intercepts of Lineweaver–Burk plots against inhibitor concentration (0–400  $\text{nmol}\cdot\text{mL}^{-1}$ ), the inhibition constant ( $K_i$ ) for Ala-Phe was estimated to be approximately 320  $\text{nmol}\cdot\text{mL}^{-1}$ . Finally, the inhibitory mechanism of Ala-Phe was investigated, revealing that Ala-Phe acts as a non-competitive inhibitor.

## Discussion

The supply of affordable and high-quality protein sources is crucial for preventing sarcopenia in an ageing population. To facilitate food intake in the elderly, it is important to process meat and fish using food-grade proteases to improve their texture. Additionally, imparting functional properties to the resulting by-products can contribute more effectively to maintaining the health of elderly individuals. In this study, we focused on the muscle tissue of the sandbar shark and investigated the ACE inhibitory activity of its protease hydrolysates. A comparative analysis of various food-processing proteases revealed that the Th treatment resulted in the least bitterness while most effectively producing ACE inhibitory activity. The bitterness assessment was performed by an untrained panel, which may introduce subjectivity; however, it was used here as a practical screening tool to support the development of palatable hydrolysates for elderly consumers. Furthermore, we identified that the ACE inhibitory effect was derived from multiple peptides, with Ala-Phe being the most effective peptide. The estimated  $K_i$  value ( $\approx 320 \text{ nmol}\cdot\text{mL}^{-1}$ ) further supports the non-competitive inhibition mechanism of Ala-Phe and indicates relatively high affinity towards ACE at physiologically feasible concentrations.

To date, numerous ACE inhibitory peptides have been discovered from various food sources and their protease hydrolysates, such as oyster, soyabean, and seaweed, which exhibit diverse structural characteristics (Sato et al., 2002; Shiozaki et al., 2010; Xu et al., 2021). The peptide Ala-Phe identified from the sandbar shark in this study has also been found in the enzymatic hydrolysates of casein (Zhu et al., 2007). In addition, previous studies have shown that Ala-Phe is transportable across Caco-2 monolayers, a model of intestinal absorption (Zhu et al., 2008), suggesting potential effectiveness in vivo; however, this was not examined in the present study.

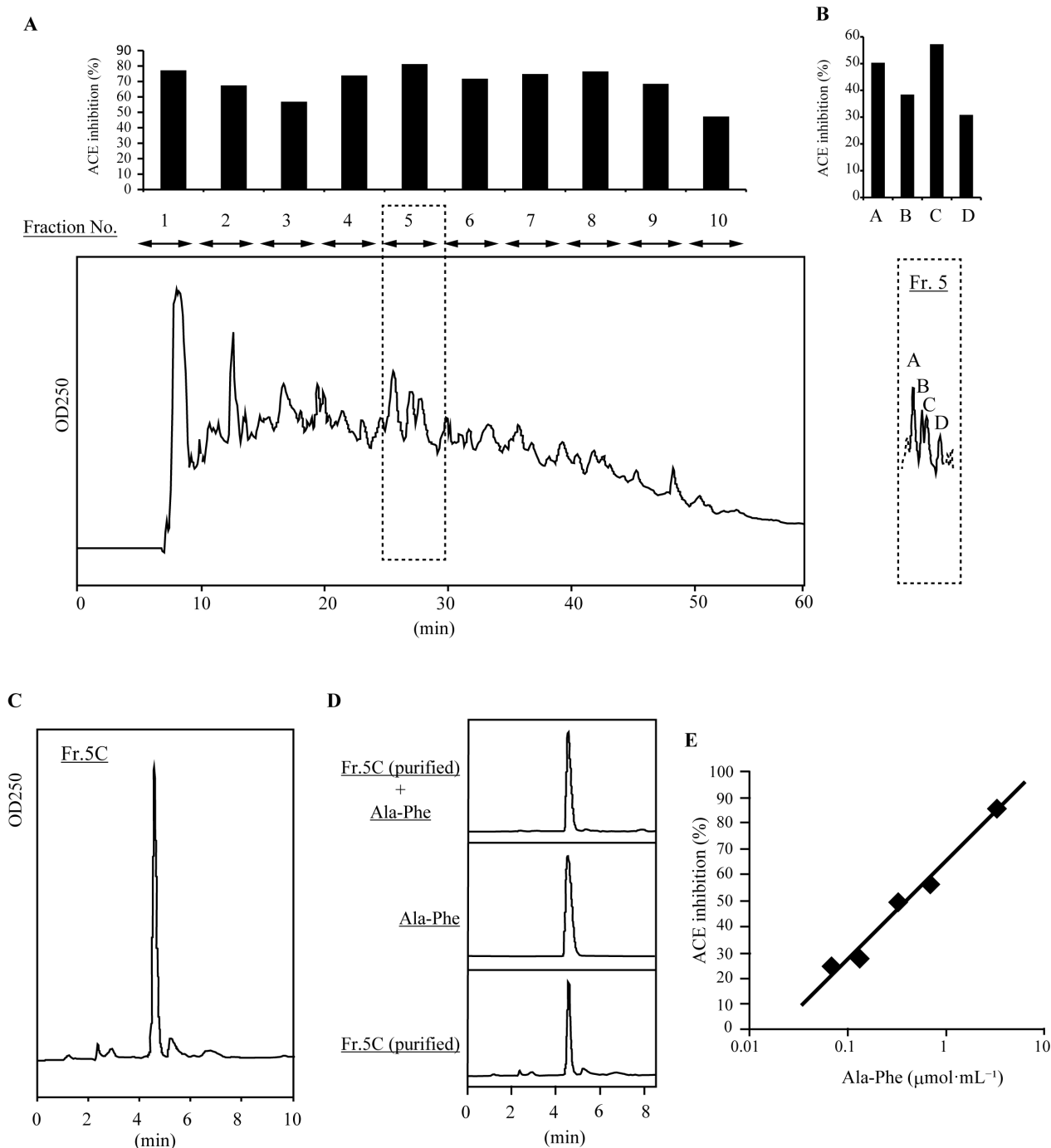


Fig. 4. Separation of crude peptide on reverse-phase column. (A) Each fraction (1–10) was individually collected using the reverse-phase column. Angiotensin-converting enzyme (ACE) inhibitory activity was evaluated for each fraction. (B) Further separation using HPLC. Four fractions were collected, and their ACE inhibitory activities were evaluated. (C) Purification of Fr. 5C. (D) Comparison of the elution time of Fr. 5C and synthesised Ala-Phe. Upper panel: Fr. 5C + Ala-Phe. Middle panel: Ala-Phe. Lower panel: Fr. 5. (E) Dose-dependent ACE inhibition by Ala-Phe.

This study differs from previous research in its main concept of utilising large sharks as a protein source for the elderly, with ACE inhibitory activity serving as a supplementary function. Shark meat is generally low in fat and has a mild taste. The sandbar shark is distributed around the Amami Islands in Kagoshima Prefecture, Japan. While shark meat is also used for processed seafood products, such as surimi, large-scale processing facilities are scarce in the island regions. Thus, distributing shark meat in its natural

form is likely the most practical approach.

Our previous research identified Val-Trp as an ACE inhibitory peptide derived from the muscle of dwarf gulper shark (*Centrophorus atomarginatus*) (Ikeda et al., 2015). However, the deep-sea dwarf gulper shark lives in the deep sea and has already been targeted by fisheries due to its high squalene content in the liver (Shiozaki et al., 2017). Furthermore, this shark is relatively small in size, which makes expanding its use

as a protein source challenging. In contrast, sandbar sharks are large, not currently targeted by fisheries in Japan, and are abundant. They are also an unused resource and relatively inexpensive. Moreover, products derived from sharks, such as chondroitin and collagen, are widely available, contributing to a generally positive public perception of sandbar shark consumption.

The sandbar shark is currently considered a nuisance species because of its detrimental impact on fisheries and is being culled with government subsidies in Japan. However, if it were to become a commercially viable resource, it could reduce the economic damage caused by these sharks. This study, therefore, has the potential to create a significant impact in multiple domains, including fisheries management, food sustainability, and elderly nutrition.

## Conclusion

This study demonstrates that processing sandbar shark using food-grade proteases can impart additional value by exhibiting ACE inhibitory activity. Given that food-grade proteases are generally regarded as safe and are primarily intended to modify food texture, this technology holds significant potential for rapid application. Since sandbar sharks are found not only in Japan but also across the Pacific region and considering the global challenges of ageing populations and hypertension, the implications of this research are substantial.

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**Conflict of interest:** The authors declare that they have no conflict of interest.

**Author contributions:** Asami Ikeda: Formal analysis, investigation, methodology, visualisation, writing-original draft. Tsugunori Maekawa: Formal analysis, investigation, validation. Kazuhiro Shiozaki: Conceptualisation, formal analysis, project administration, supervision, writing-original draft, writing-review and editing.

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