

## Production of Water Soluble Antioxidative Plastein from Squid Hepatopancreas

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Edited by J. Sekiya, Kyoto Univ., and accepted December 27, 2003 (received for review August 22, 2003)

**Abstract:** To utilize a typical squid processing by-product, hepatopancreas was enzymatically hydrolyzed, then subjected to a plastein reaction. The substrate concentration that gave the highest plastein yield was 30% by using Alkalase. The optimum pH for the plastein synthesis with Alkalase ranged from 6 to 9. The dominant molecular size of the formed plastein was about 2000. Alkalase mediated plastein reaction seemed beneficial for enriching aspartic acid and glutamic acid, and eliminating hydrophobic amino acids such as phenylalanine, tyrosine, leucine, and isoleucine. Despite the heat pretreatment, hydrolysate powder and plastein retained their antioxidative affect, but a discouraging drawback of the hydrolysate was an unacceptable stimulating taste. For this reason, plastein was considered to be a much better choice to use as an antioxidant because it was tasteless. Plastein from squid hepatopancreas may be a useful antioxidant because it is stable against heat, dissolves easily in water and suppresses the prooxidative effect of metals on lipid oxidation.

**Key words:** plastein, antioxidant, squid, alkalase

### 1 Introduction

Squids are one of the most highly consumed sea foods for Japanese and many other Asian peoples. To decrease by-products and waste from squid, various ways of utilization from highly value added products to a fairly low value added but with a large mass demand must be developed. At present, the only available utilization of by-products and waste from squid on a large scale has been the production of feeds, but the market for feeds is limited. For this reason, developing a more effective way to utilize squid related by-products and waste is becoming more and more important. Vast excess waste, mostly from the hepatopancreas of squid

has been a burden for those processing companies. Equipment investment for reducing by-products and waste might become possible if we could develop value added products. Food additive uses should have the potential to be highly value added.

The purpose of this paper is to show the potential benefits of utilizing an enzymatically modified squid hepatopancreas protein as a water soluble and heat stable antioxidant.

### 2 Experimental

#### 2.1 Materials

Fresh squids (*Todarodes pacificus*) were obtained

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from a market in Obihiro city, Hokkaido Japan. Hepatopancreases were separated and homogenized with a juicer mixer. Pasteurization was done at 105°C for 30 min and then homogenized again. The crude protein content of the homogenate was determined according to the Micro Kjeldahl Method.

## 2·2 Proteolytic Hydrolysis

A five times volume of water was added to the homogenate, then heated up to 50°C. Alkalase 2.4L (Novo Nordisk, Denmark) corresponding to 2% of crude protein contained in the homogenate was added to start the hydrolytic reaction. After 19 h, the reaction was terminated by increasing the temperature to 90°C, and maintaining it for 30 min. After centrifugation (10 min at 12,000 × g under 5°C), the supernatant except the oily layer was collected, then filtered through an ADVANTEC No.5 filter paper (ADVANTEC Co., Ltd., Tokyo, Japan) with a Celite 545 filter aid (Wako Pure Chemical Industry Co., Ltd., Japan). The filtrate was desalted with an Electric Dialyzer (Model DU-Ob, Nippon Densui Co., Ltd., Japan) with Seremion CM-V and AM-V (Asahi Glass Co., Ltd., Japan), then freeze dried (Model TFD-550-8 Takara-Seisakusyo Co., Ltd., Japan).

## 2·3 Plastein Reaction

### 2·3·1 Determination of Optimum Substrate-Enzyme Ratio

To each 0.50, 1.00, 1.25, 1.50, 1.75, 2.00, and 2.50 g of the hydrolysate dried powder, 5 mL distilled water was added in order to prepare 10, 20, 25, 30, 35, 40, and 50% (w/v) substrate concentration solutions, respectively. After adjusting the pH to 8.0, 2 mL was dispensed for Alkalase mediated plastein reaction and another 2 mL for control from each individual substrate solution. The ratio of Alkalase to substrate was 1:100 (w/w). The plastein reaction was conducted under 55°C in a reciprocal manner shaking for 24 h, then increased to 90°C and kept of that temperature for 30 min to terminate the reaction. They were cooled to the ambient temperature, and 2.0 mL of 20% trichloric acid solution was added to precipitate the plastein. Centrifugation was done at 3,600 × g under 5°C for 10 min to recover the plastein, then rinsed 3 times with 10% trichloric acid. All the filtrates were combined and the Lowry method was conducted. Plastein synthetic yield (%) was calculated by subtracting the amount of Lowry method

positive substances in the filtrate from that of control, then dividing by the Lowry method positive substances in the control and multiplied by 100 to express as % yield.

### 2·3·2 Determination of Optimum pH

To 6 g of hydrolysate dried powder, 20 mL distilled water was added. After mixing, 2 mL was dispensed individually in order to prepare pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0 reaction mixtures. Adjustments were done with NaOH and HCl. The ratio of Alkalase to substrate was 1:100 (w/w). Plastein reaction and plastein synthetic yield (%) calculation were done as mentioned above.

## 2·4 Determination of Molecular Size Distribution

Molecular size distributions were determined by a 4  $\Phi$  × 30 cm length G-3000 column (TOSOH, Japan) equipped HPLC (TOSOH SC-8020, Japan) analysis with 0.1% TFA containing 45% acetonitrile mobile phase with UV detection (TOSOH 8020 detector, Japan) at 280 nm. The flow rate was 0.3 mL/min. The column temperature was maintained at 40°C.

## 2·5 Amino Acid Composition Analysis

For the amino acid composition analysis, the PTC pre-labeled method was used on HPLC (TOSOH SC-8020, Japan) equipped with a UV detector (TOSOH SC-8020, Japan) and ODS-80Ts 4  $\Phi$  × 25 cm + 4  $\Phi$  × 15 cm columns (TOSOH, Japan) connected in tandem. Two kinds of mobile phases, i.e. (eluent A) acetonitrile: pH 5.6 60 mM acetic acid buffer (6:94,v/v) and (eluent B) acetonitrile: pH 7.0 60 mM acetic acid buffer (3:2,v/v) were utilized to effect separations of a wider range of individual amino acids. Separation was conducted at 280 nm UV detection under 45°C at a flow rate of 1.0 mL/min. Gradient elution conditions were B:5% at 7.5 min, B:19% at 25 min, B:60% at 45 min, and B:100% at 60 min.

## 2·6 Evaluation of the Plastein as an Antioxidant

Plastein was prepared under the optimum condition as determined by the aforementioned pH conditions. Molecular weight under 1000 was removed with a cellulose ester membrane tube MWCO:1000 (Spectrum Laboratories Inc., USA). Plastein with a molecular weight over 1000 was freeze dried.

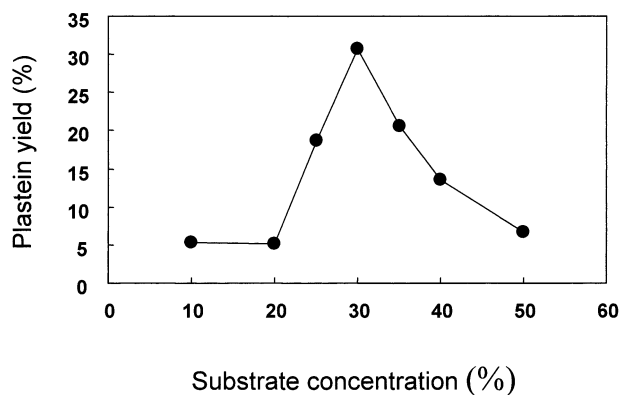
Antioxidative activity of the plastein was evaluated in 4 way conditions on sample solution shown in **Table 1** (1). The 4 way conditions were, A:without hemin and without heat pretreatment of the antioxidants, B:without hemin but with 2 h heat pretreatment of the antioxidants at 95°C, C: with hemin but without heat pretreatment of the antioxidants, D:with hemin and with 2 h heat pretreatment of the antioxidants at 95°C. Screw capped vials or test tubes with glass balls on them were used to avoid oxidation during the oxidative rate measurements. Oxidations were monitored under 40°C by picking up each reaction tube at certain times. Reaction tubes were prepared as follows. Linoleic acid was mixed into the sample solution to give a 20 mM concentration, then plastein corresponding to 0.1 weight % against linoleic acid was mixed. The same weight % of  $\alpha$ -tocopherol, bovine serum albumin, and hydrolysate dried powder were also examined instead of plastein to compare the antioxidative effect. Systems with hemin (10 ppm against linoleic acid) and without hemin were also compared under the same condition. Following the method invented by Mitsuda (2), the oxidative rates were monitored at 500 nm absorbance 3 min after mixing the 100  $\mu$ L sample solution with 2 mL of ethanol, 2.7 mL of distilled water, 0.1 mL of 30% ammonium thiocyanate, and 0.1 mL of 3.5% hydrochloric acid containing 20 mM ferric sulfate. In another method, the thiobarbituric acid reactive substance value (TBA-V) measurement (3) was employed. Absorbance at 532 nm was monitored for TBA-V after mixing the 50  $\mu$ L sample solution with 0.8 mL of distilled water, 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid buffer (pH 3.5), and 1.5 mL of 0.8% TBA, then increased to 95°C for an hour, and cooled in a refrigerator for one hour. Hemin

dissolved in 10% KOH was used for the metal prooxidation.

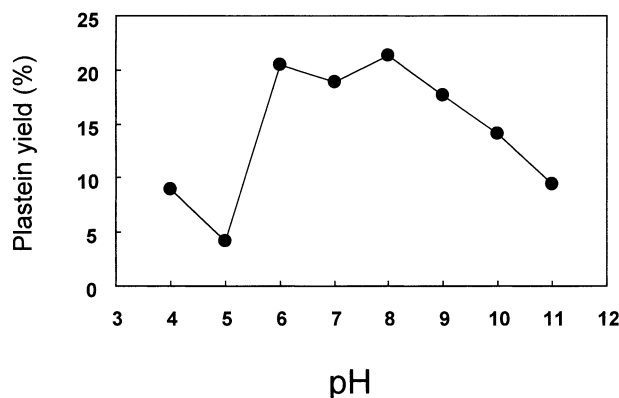
### 3 Results and Discussion

#### 3.1 Plastein Yield and Molecular Size Distribution

The substrate concentration that gave the highest plastein yield was 30% as shown in **Fig. 1**. Substrate concentrations below 20% gave an extremely poor yield. The optimum pH for Alkalase mediated plastein synthesis was not clear as shown in **Fig. 2**. We should say that a pH from 6 to 9 is recommendable for the plastein reaction when using this enzyme on the squid hepatopancreas hydrolysate powder as substrate. A wide optimum range should be beneficial for an indus-



**Fig. 1** Relationship between Plastein Yield and the Substrate Concentration of Squid Hepatopancreas Hydrolysate.



**Fig. 2** Relationship between Plastein Yield and the pH of Squid Hepatopancreas Hydrolysate.

**Table 1** Composition of the Sample Solution for the Evaluation of Antioxidative Activity of the Prepared Plastein.

99.5% Ethanol	10.0 mL
50 mM Phosphate buffer	10.0 mL
Linoleic acid	0.14 g
Sample	0.14 mg
Distilled water	$\alpha$ mL
Hemin	1.4 $\mu$ g
Total	25.0 mL

trial process, but at the same time, careful suppression of undesirable microbes is necessary.

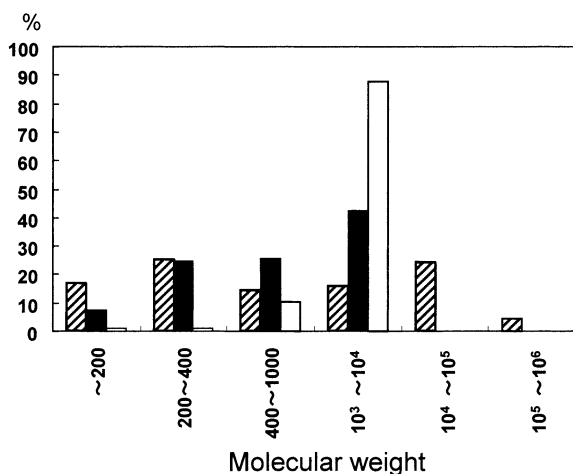
The most dominant molecular size of the formed plastein was about 2000 (data not shown). From the molecular size distribution bar graph shown in **Fig. 3**, we can see that the peptide synthetic reaction i.e. the plastein reaction was successfully done.

### 3·2 Amino Acid Compositions

The Alkalase mediated plastein reaction seemed beneficial for enriching aspartic acid and glutamic acid as shown in **Table 2**. We observed the same results (enrichment of aspartic acid and glutamic acid) when scallop mid gut gland hydrolysate powder was used as substrate for the plastein reaction (Kasai, D., Unpublished data). On the contrary, hydrophobic amino acids that are responsible for the occurrence of bitterness (4) such as phenylalanine, tyrosine, leucine, and isoleucine decreased, and so did the scallop mid gut gland hydrolysate powder (Kasai, D., Unpublished data). In fact, the bitterness in the hydrolysate disappeared after it turned into plastein.

### 3·3 Antioxidative Activity

In contrast to the wealth of information pertaining to the antioxidative peptides derived from food itself or from by-products (5-12), there has been no report on the antioxidative peptides derived from the internal

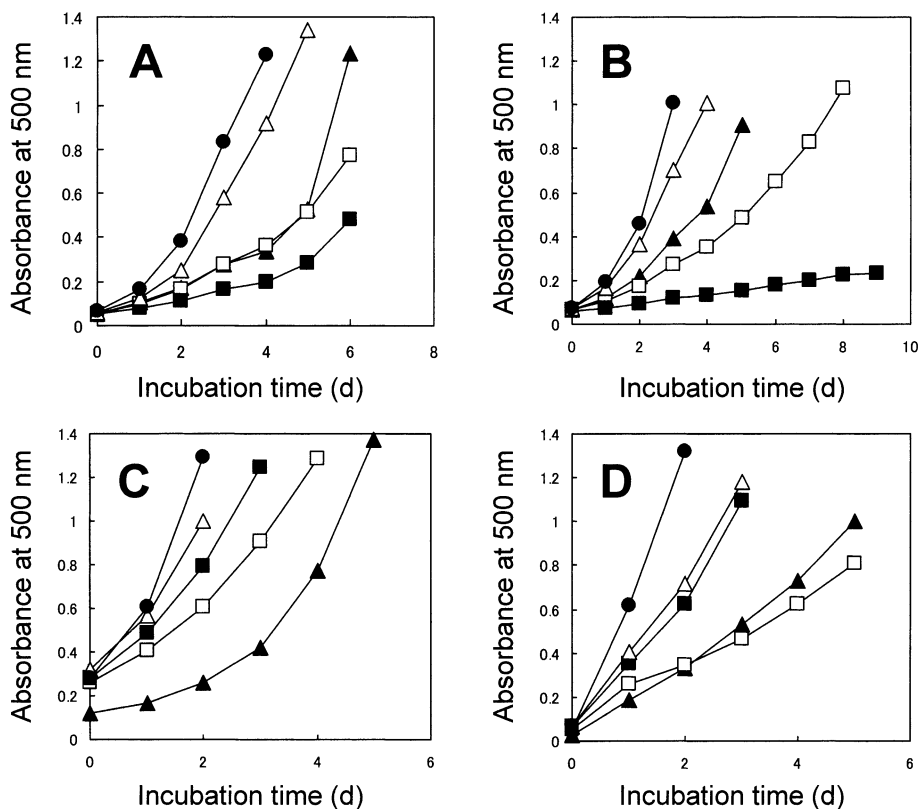


**Fig. 3** Peptide Molecular Weight Distribution of the Squid Hepatopancreas Raw Material, the Hydrolysate, and the Plastein.  
 ▨: Raw, ■: Hydrolysate, □: Plastein

**Table 2** Amino Acid Compositions of Squid Hepatopancreas and Derived Products.

Amino acid	Raw	Hydrolysate	Plastein
Asp	7.95	7.53	14.39
Glu	12.31	11.90	14.57
Ser	5.38	6.38	7.50
Gly	8.33	8.94	12.19
His	1.84	1.28	1.40
Arg	3.99	2.97	5.22
Thr	5.22	5.55	7.19
Ala	7.47	10.32	5.24
Pro	5.31	4.87	8.73
Tyr	2.96	1.57	1.06
Val	6.49	6.41	4.04
Met	2.92	2.51	1.25
Cys	0.00	0.00	0.00
Ile	6.24	5.63	3.21
Leu	8.62	9.65	3.49
Phe	4.00	3.24	1.55
Lys	6.24	7.19	8.65
Total	100.00	100.00	100.00

organs of marine animals, even though a great excess of them have been a burden to sea food processing companies. For this reason, we investigated the antioxidative activities of hydrolysate powder and plastein derived from squid hepatopancreas. Despite the heat pretreatment of the antioxidants examined, hydrolysate powder and plastein from squid hepatopancreas sustained their antioxidative effects and showed much better suppression of lipid oxidation than other antioxidative substances examined, as shown in B on **Figs. 4** and **5**. Surprisingly, the hydrolytic powder increased its antioxidative effect after heat pretreatment as seen in the same figure. Unfortunately, we do not have any idea why this occurred. The only but serious discouraging drawback of this hydrolysate was an unacceptable stimulating taste. For this reason, plastein should be a much better choice to use as a heat stable antioxidant because it is tasteless. In contrast to the hemin free systems, hydrolysate powder had a greater decline in its antioxidative effect than plastein (Comparison between B and D in **Figs. 4** and **5**) in hemin added system. Alpha-tocopherol was effective under hemin added conditions. But when it was subjected to heat pretreatment, the antioxidative effect of the  $\alpha$ -tocopherol declined and became



**Fig. 4** Time Courses of Oxidation Monitored by Ammonium Thiocyanate with Ferric Sulfate Reaction Method.

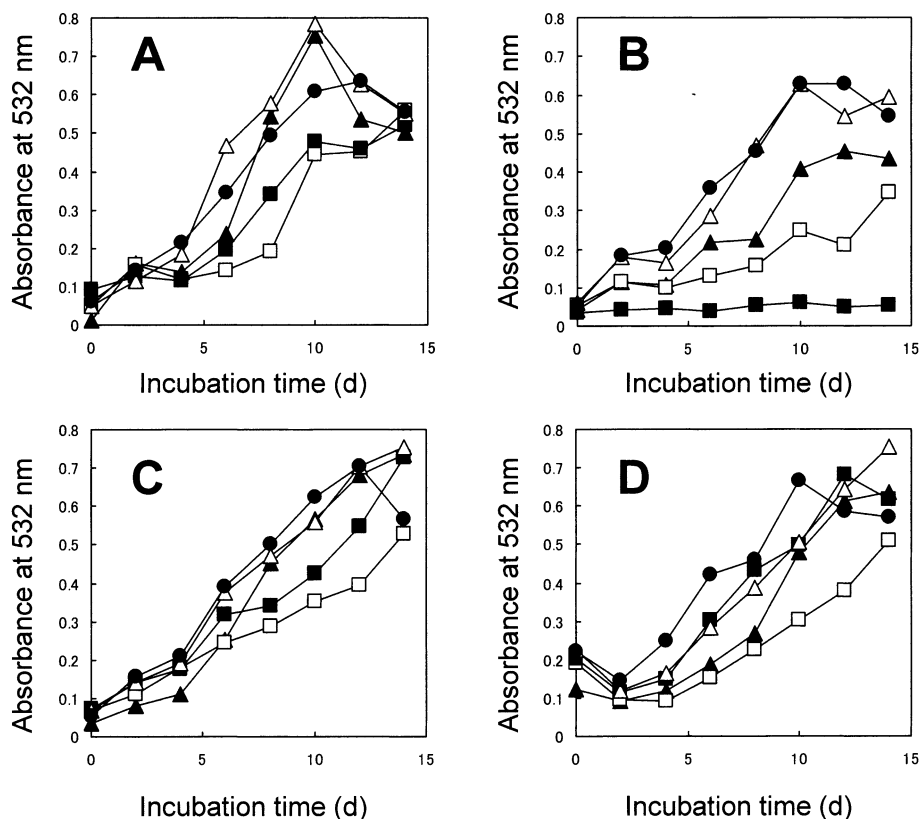
●: Control, ▲:  $\alpha$ -tocopherol,  $\triangle$ : Albumin, ■: Hydrolysate,  $\square$ : Plastein  
 A: without heating and without hemin,  
 B: without hemin but with 2 h heat pretreatment of the antioxidants,  
 C: with hemin but without 2 h heat pretreatment of the antioxidants,  
 D: with heating and with hemin

comparable to the effect of plastein. Plastein would dissolve far easier in foods than  $\alpha$ -tocopherol because plastein prepared in the aforementioned manner is water soluble so that it might be more beneficial to use plastein than tocopherol such as in juicy meat foods.

Muramoto and Hatakeyama (13) reviewed the antioxidative effects of various kinds of peptides and pointed out that peptides rich in antioxidative amino acids should be one reason to exert the antioxidative activity but with some exceptions. And another reason should be the affinity of peptides for prooxidants. They also mentioned that there would be many other reasons why peptides with certain amino acid sequences exert antioxidative activity. They proposed a combination mechanism of chelating effects on metals especially by histidine, synergetic effects with other components, a

quenching effect on active oxygen, a trapping effect on singlet oxygen, a scavenging effect on hydroxyl radicals, and the forming of electron transferring complexes which may contribute to stabilizing the lipid oxidative compounds.

One thing we may have to keep in mind is the condition of hepatopancreas such as freshness and the physiological status when captured. They may vary and should affect on the peptide composition of its hydrolysate. Therefore, it may also affect the characteristics of the plastein produced from those hydrolysates. For this reason, it is recommendable to carry out the same experiments on hepatopancreas of different status.



**Fig. 5** Time Courses of Oxidation Monitored by TBA-V.  
 ●: Control, ▲:  $\alpha$ -tocopherol, △: Albumin, ■: Hydrolysate, □: Plastein  
 A: without heating and without hemin,  
 B: without hemin but with 2 h heat pretreatment of the antioxidants,  
 C: with hemin but without 2 h heat pretreatment of the antioxidants,  
 D: with heating and with hemin

#### 4 Conclusion

Plastein from squid hepatopancreas may be a useful antioxidant because it is stable against heat, tasteless, dissolves easily in water and may suppress the prooxidative effect of metals on lipid oxidation.

#### Acknowledgment

Thanks are due to Mr. Masato Ohmae for the preliminary experiments carried out prior to this study. This work is a part of “Developing highly value-added technology based on the results of academic research, development of technologies for pre-treating industrial waste to recover and reprocess high-quality raw materials” supported by JST (Japan Science and Technology Corporation).

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