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南極蝦加工利用研究(一)

Studies on Processing and Utilization of Antarctic Krill (I)

目　錄

Studies on the Proteases of Antarctic Krill *Euphausia superba*–Activities and Stability

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SUMMARY

Crude extract and purified krill proteases, tentatively called proteases A_1, A_2, B, C and D were used for this study. A_2, B, C and D belong to trypsin-like enzymes, while the substrate specificity of A_1 has not been fully characterized. The specific activity of the crude extract was an order of magnitude higher than samples from other sources. The purified proteases except for protease D, had lower proteolytic activity than the mammalian enzymes. The proteolytic activity decreased in the following order: D>C>A_1>B>A_2. Protease D had proteolytic activity in the same order of magnitude as those of bovine trypsin and chymotrypsin. On the other hand the esterase actvities of C and D were in the same order of magnitude as that of bovine trypsin, whereas A_2 and B had much lower esterase activities.

Optimal pH of the crude extract was 8 and optimal temperature around 50°C. The crude extract was labile at higher temperatures. The activity decreased remarkably when incubated at temperatures above 40° C for 10 min. However, it was stable if stored frozen, lyophilized or in saturated ammonium sulfate. Studies on stability of krill proteases revealed that the crude extract should be stored at -20° to -30° C, whereas proteases in either saturated ammonium sulfate solution or lyophilized form could be stored at 5°C. The latter also could be stored at 20° C with only minor loss of activity.

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INTRODUCTION

An increasing demands of the growing population in the world for improved diet would eventually result in a shortage of foods, more specifically proteins. Therefore, providing human being with high-quality protein foods is one of the main problems of today. According to estimations made by different investigators, the total resources of Antarctic krill in the South Ocean amounts to an astronomical figure of 0.6 to 2 billion tons. In addition, it has been reported that the biomass of the Antarctic krill is as high as 10 to 15 kg/m^3. High abundance and formation of massive concentrations point to strong possibilities of the commercial utilization of krill resources. The potential annual catch estimated by FAO is 50 to 70 million tons[1]. In view of such abundant krill resources and its strong possibility of serving as protein source for human consumption, our government had sent Hai-Kung Research Vessel to the Antarctic Ocean for exploration of resources in that area in December 1975 and brought back 136 tons of krill. The event, a milestone in the history of Chinese fisheries, thus initiated the active participation of Chinese in the exploitation of Antarctic resources.

In utilization of krill as a new source of portein food one faces the problem of rapid spoiling of krill resulting from blackening and autolysis presumably caused by tyrosinase and proteases, respectively[2,3]. It was reproted that within two hours on deck krill turned pale in color, lost transparency and became soft and fabby, with the cephalothorax turning dark. Judging from the unusual rapid autolysis of krill, it is suggested that krill may contain some highly active proteases. Studies on the krill proteases could provide useful information for preservation of fresh krill on the one hand and might make the enzyme practically applicable to food processing or medical field on the other hand. In this paper we present the proteolytic and esterase activities of purified proteases from krill as compared with that of some commercially available proteases. Stabilities of the crude proteases of krill under different storage conditions are also described.

MATERIALS and METHODS

Materials

Trypsin, chymotrypsin, papain, casein, hemoglobin, bovine serum albumin, benzoyl-L-arginine ethyl ester (BAEE) and p-toluenesulfonyl-L-arginine methyl ester (TAME) were purchased from Sigma Chemical Company. All other chemicals were reagent grade.

Antarctic Krill

Freshly frozen krill, *Euphausia superba*, which were captured in January 1976 at the Antarctic Ocean and stored frozen at-40°C was provided by Taiwan Fisheries Research Institute and stored at-30°C until use.

Extraction of Proteases

Preliminary experiment showed that 0.1M phosphate buffer, pH 7.0, was superior to other three extraction media tested for extraction of proteases from krill. Therefore phosphate buffer was employed. One hundred gram of frozen krill were suspended in 200 ml of 0.1M potassium phosphate buffer, pH 7.0 and homogenized in a Waring blendor. The insoluble material was removed by centrifugation at 7500×g for 20 min. The supernatant was dialyzed against the same buffer. All steps were performed at 5°C.

Assay of Proteolytic Activities

1. Casein as substrate: Caseinolytic activity was measured according to Kunitz method[4]. One unit of enzyme activity was defined as the amount of protease caused an increase in absorbance at 280 nm of 1.00 in 10 min. Specific activity was expressed as unit per mg protein.

2. Hemoglobin as substrate: The method described by Anson was used[5]. The denatured hemoglobin was digested for 10 min at 37°C, the reaction was stopped by the addition of 5% trichloroacetic acid, and the nondigested hemoglobin was removed by filtration. The amount of split products remaining in solution was determined spectrophotometrically at 280 nm. Definition of

units and specific activity was the same as that for caseinolytic assay.

3. Bovine serum albumin as substrate: Denatured bovine serum albumin was used as substrate. 1 g of bovine serum albumin was dissolved in 100 ml of 0.1M potassium phosphate buffer, pH 8.0 and heated in a boiling water bath for 15 min. The assay procedure was essentially the same as that of hemoglobinolytic assay.

Assay of Trypsin Esterase Activities.

1. BAEE as substrate[6]: 2.8 ml of 1 mM BAEE in 50 mM Tris-HCl, pH 8.0 containing 0.02 M $CaCl_2$ was measured into a cuvette. 0.2 ml of properly diluted enzyme solution was added at zero time and mixed immediately. Absorbance at 253 nm was taken at 30-second intervals. One unit of BAEE activity was defined as the amount of trypsin resulted in an absorbance increase of 1.00 per 10 min. Specific activity was expressed as unit per mg protein.

2. TAME as substrate[7]: 2.6 ml of 46 mM Tris-HCl, pH 8.1 containing 11.5 mM $CaCl_2$ was measured into a cuvette followed by 0.3 ml of 10 mM TAME. 0.1 ml of properly diluted enzyme solution was added at zero time and mixed immediately. Absorbance at 247 nm was taken at 30-second intervals. One unit of TAME activity was defined as the amount of trypsin resulted in an absorbance increase of 1.00 per 10 min. Specific activity was expressed as unit per mg protein.

Protein Determination

Protein was determined by the Lowry method[8] using bovine serum albumin as a standard.

RESULTS and DISCUSSION

Specific Activity of the Crude Extract of Krill

A comparison of the specific activity of the crude extract of krill with that of some aquatic organisms is shown in Table 1. The specific activity of the crude extract of krill was one order of magnitude higher than that of the

Table I. Protease Activities in Some Aquatic Organisms

Speceis	*Euphausia superba* (krill)	*penacus japonicus* (tiger shrimp)	*Macrobrachium rosenbergii* (giant freshwater prawn)		*Cyprinus carpio* (LINNE) (carp)
	whole krill	muscle	cephaloth-orax	abdomen	muscle
Specific activity (A 280nm/10min /mg protein)	3.83×10^{-1}	1.73×10^{-2}*	3.09×10^{-2}	4.20×10^{-3}	3.93×10^{-2}**

* Iwata, K., et al. Bull. Jap. Soc. Sci. Fisheries. **40**, 201-209 (1974)

** Iwata, K., et al. Bull. Jap. Soc. Sci. Fisheries. **38**, 1325-1337 (1973)

three samples from other sources.

Optimal pH and Temperature of the Crude Extract

The pH optimum was 8 as shown in Fig. 1. However the hemoglobinolytic activity distributed over a wide range of pH with a shoulder around pH 5.5-

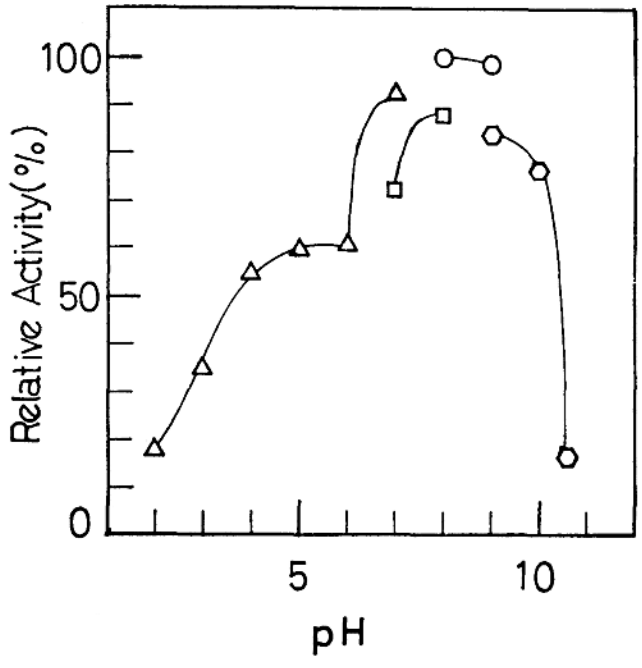

Fig. 1. pH-Activity Profile of the Crude Extract of Krill
—△— Citrate-phosphate buffer, —□— phosphate buffer,
—○— Tris-HCl buffer, —⬡— glycine-NaOH buffer.

6.0. The result seemed to indicate that the crude extract contained both acidic, neutral and alkaline proteases. The data presented in Fig. 2 indicated that the optimal temperature was about 50°C. It is worth noting that the crude extract showed 5% of its maximal caseinolytic activity at 0-5°C. This might account for the rapid autolysis occurred in thawing the frozen krill.

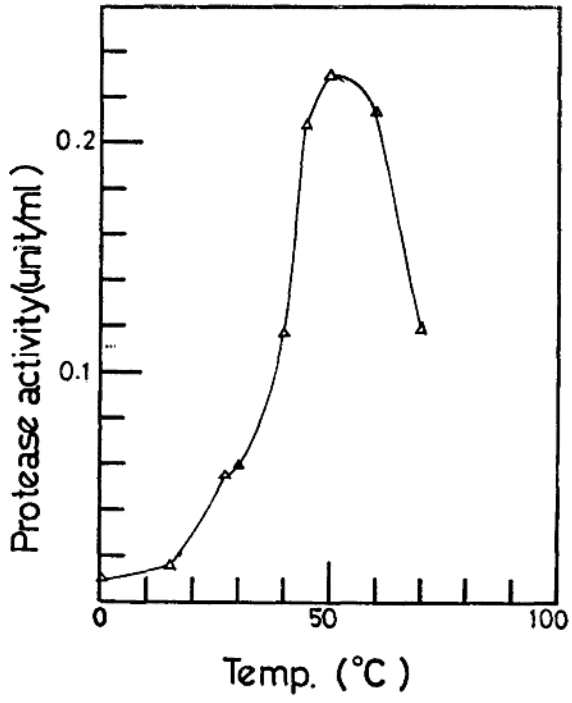

Fig. 2. Temperature-Activity Profile of the Crude Extract of Krill

Proteolytic Activity of Purified Krill Proteases

The proteolytic activity of the crude extract of krill was separated into five proteolytic enzymes, called proteases A_1, A_2, B, C, and D. A_1, B, C, and D were purified to homogeneity, although A_2 was only partially purified. A_2, B, C and D were identified as trypsin-like enzymes, while A_1 has not been fully characterized[9]. The five purified proteases were examined for their specific activity toward three natural protein substrates—casein, hemoglobin and bovine serum albumin. The proteolytic activity of four commercially available proteases which are commonly used in food manufacture and medical field were also determined for comparison. The results shown in Table II

Table Ⅱ. Comparison of the Activities of Purified Krill Proteaes with Four Selected Proteases

Substrate	Specific Activity (unit/mg protein)								
	Krill proteases					Bovine trypsin	Bovine chymo-trypsin	Hog pepsin	Papain
	A_1	A_2	B	C	D				
Casein	2.56	0.84	2.00	4.08	9.18	11.85	16.89	—	2.24
Hemoglobin	10.36	—	5.40	—	13.03	45.61	20.53	54.34	1.50
Bovine serum albumin	3.70	—	—	—	6.32	3.31	1.87	11.99	1.86

indicated that all of the purified krill proteases except for protease D had lower proteolytic activity than the mammalian enzymes. Protease D had activity in the same order of magnitude as those of bovine trypsin and chymotrypsin. The caseinolytic activity of the purified krill proteases decreased in the following order: $D>C>A_1>B>A_2$.

Esterase Activities of Purified Krill Proteases

Since krill proteases A_2, B, C and D were identified as trypsin-like enzymes, their activity toward trypsin specific substrates, BAEE and TAME, were compared with that of bovine trypsin. The results are shown in Table III. The esterase activities of C and D were in the same order of magnitude as that of bovine trypsin, whereas proteases A_2 and B had much lower esterase

Table Ⅲ. Esterase Activities of Purified Krill Protease and Bovine Trypsin

Substrate	Specific Activity (unit/mg protein)				
	Krill Proteases				Bovine Trypsin
	A_2	B	C	D	
BAEE	4.35	2.34	182.2	137.7	63.1
TAME	8.70	7.25	132.7	173.5	340

activities. The lower esterase activity of A_2 cannot be simply explained by the presence of impurity. A_2 and B also had lower proteolytic activities as shown in Table II. Further work is needed to be done in order to clarify this point.

Even the purified krill proteases showed lower proteolytic activity than the four commercial proteases, it is worth considering the practical applications of the krill proteases in food processing and medical field because krill extract showed high specific activity and the resources of Antarctic krill are tremendously abundant.

Stability of the Crude Proteases of Krill

1. In liquid form: The crude proteases were labile at higher temperatures. The activity decreased remarkably when incubated at temperature above 40°C for 10 min as shown in Fig. 3. Fig. 4 shows the caseinolytic activities of

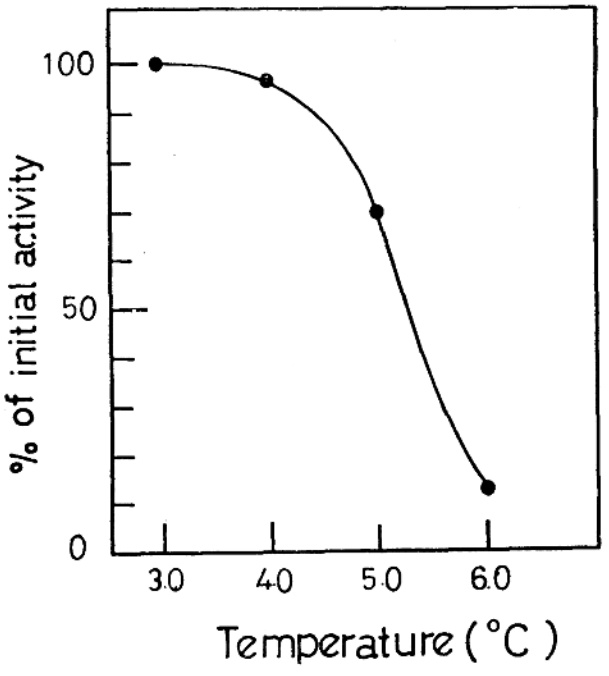

Fig. 3. Thermostability of the Crude Extract of Krill

The crude extract was preincubated at various temperatures for exactly 10 min, cooled to 0°C, and assayed for caseinolytic activity.

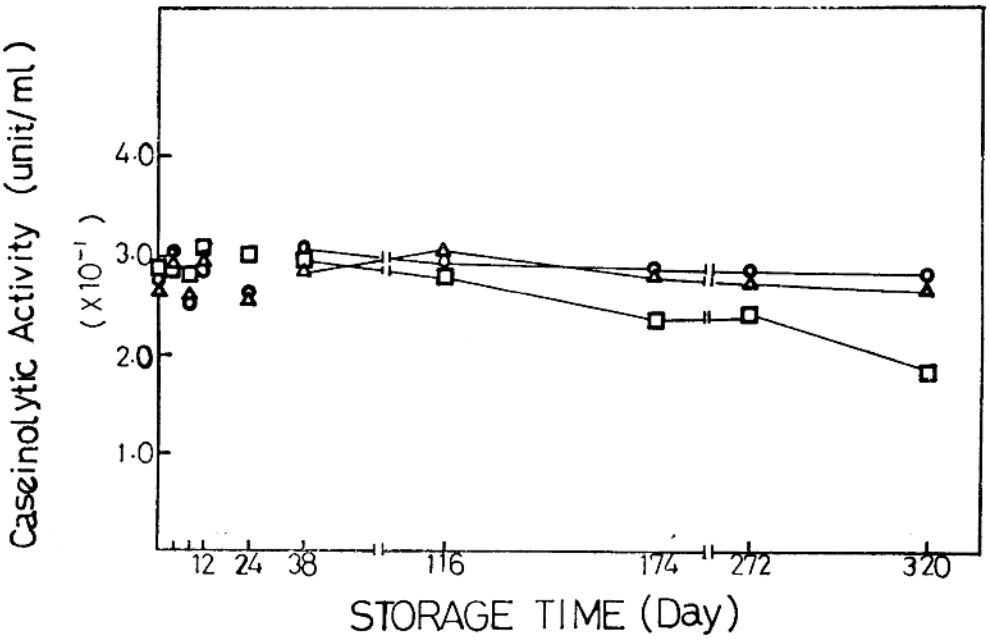

Fig. 4. Stability of the Crude Extract of Krill

—□— 5°C, —△— -20°C, —○— -30°C.

the crude extract after storage at -30°, -20° and 5°C for various time intervals. The loss of activity was only 10% after 320 days of storage at -30° and -20°C, whereas 36% loss was observed for the sample stored at 5°C. The results showed that the crude extract was quite stable at -30° to -20°C.

2. Lyophilized crude proteases: Dialyzed crude extract was lyophilized and stored at 20°, 5° and -20°C. The caseinolytic activities of each sample assayed after different periods of storage are shown in Fig. 5. The results indicated that lyophilized crude extract had 84%, 95% and 98% of activities left after storage for 320 days at 20°, 5° and -20°C, respectively.

3. Crude enzymes saturated with ammonium sulfate: The crude extract was fractionated with ammonium sulfate to obtain three ammonium sulfate

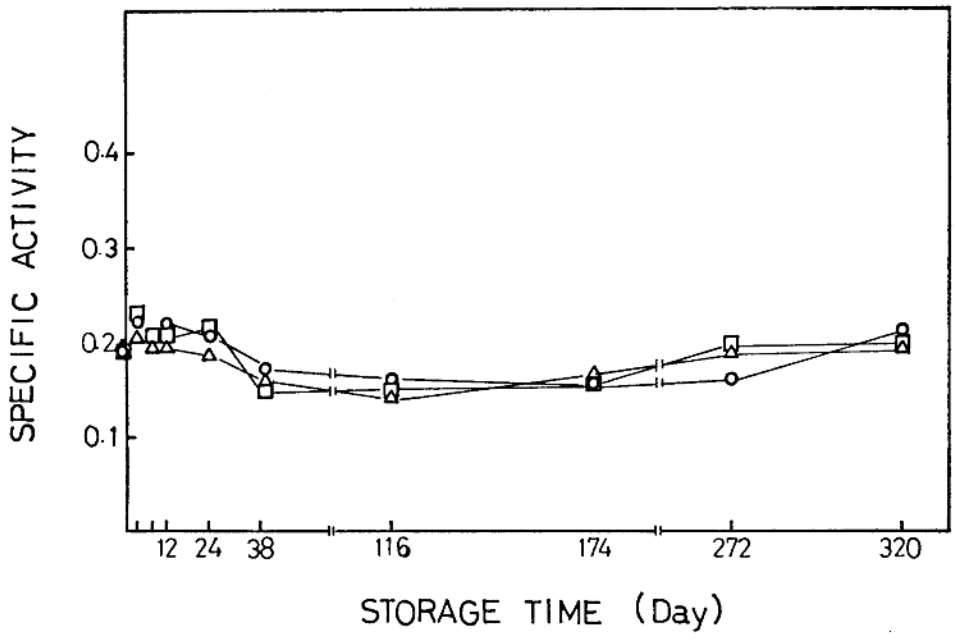

Fig. 5. Stability of the Lyophilized Crude Krill Proteases

—□— 20°C, —△— 5°C, —○— -20°C.

fractions, 0-50%, 50-75% and >75%. Each fraction was divided into two parts and stored at 5° and -20°C. An aliquot of each sample was taken and the specific activity determined after different periods of storage. As shown in Fig. 6, 81% activity of 0-50% faction and 92% activity of 50-75% fraction survived from 320 day storage at 5°C, whereas almost insignificant loss of activity was observed for samples stored at -20°C.

4. Lyophilized ammonium sulfate fractions: Both 0-50%, 50-75% and >75% ammonium sulfate fractions were divided into three parts, stored at 20°, 5° and -20°C, and assayed for caseinolytic activity after different periods of storage. The results are shown in Fig. 7. The 50-75% fraction was more stable than the 0-50% fraction. After storage at 20°C for 320 days, the former had 83% activity survived, whereas the latter had only 67% activity left.

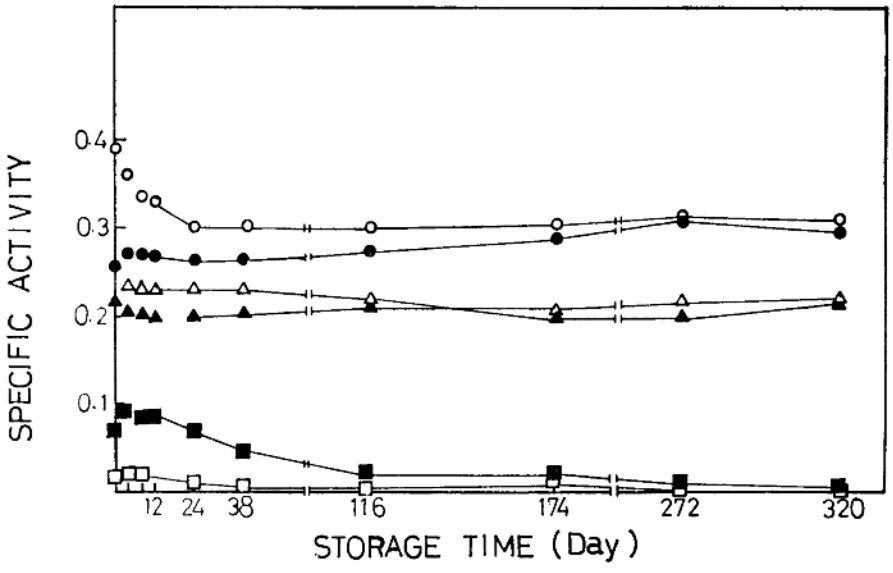

Fig. 6. Stability of the Crude Krill Proteases Saturated with Ammonium Sulfate

	5° C	-20° C
0-50% ammonium sulfate fraction:	—○—	—●—
50-75% ammonium sulfate fraction:	—△—	—▲—
> 75% ammonium sulfate fraction:	—□—	—■—

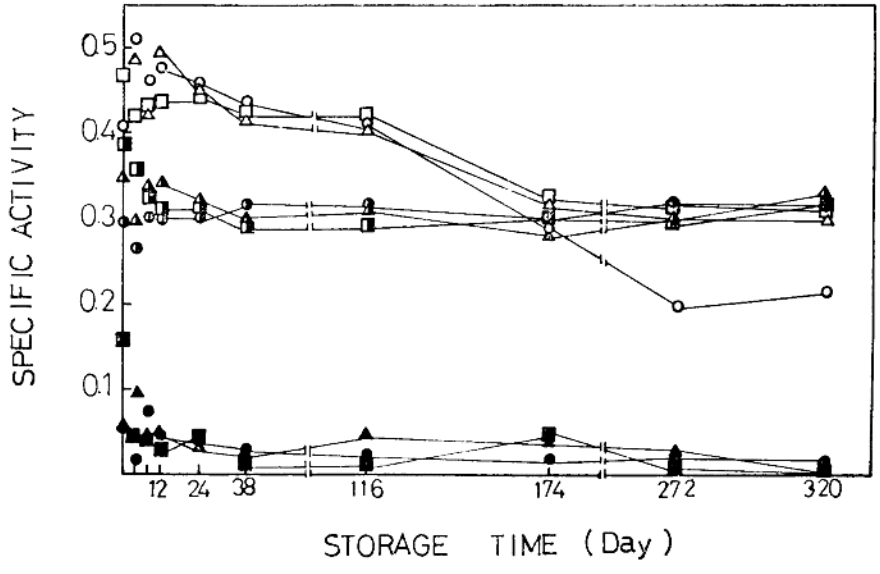

Fig. 7. Stability of the Lyophilized Ammonium Sulfate Fractions of Krill Proteases

	-20° C	5° C	20° C
0-50% ammonium sulfate fraction:	—○—	—△—	—□—
50-75% ammonium salfate fraction:	—◑—	—◭—	—◨—
> 75% ammonium sulfate fraction:	—●—	—▲—	—■—

The following conclusion can be drawn from the stability experiments. Krill proteases in liquid form should be stored at -20° to -30°C, while that in saturated ammonium sulfate solution and in lyophilized form could be stored at 5°C. The latter also could be stored at 20°C with minor loss of activity.

ACKNOWLEDGEMENTS

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南極蝦蛋白酶之研究

——活性及安定性——

陳慶三、高淑文、顏聰榮

試驗2%氯化鈉、2%氯化鉀、水及0.1M磷酸緩衝液（pH 7.0）等四種溶液的抽出效果顯示0.1M磷酸緩衝液最適於自南極蝦抽取蛋白酶。粗酵素經純化後獲得五種蛋白酶，命名爲A_1、A_2、B、C和D。A_1、B、C及D等經鑑定爲均質，A_2之純度估計約80%以上。依其基質特異性將A_2、B、C及D歸類爲胰蛋白酶。A_1之特異性尚未充分瞭解。

D之活性與胰蛋白酶及胰凝乳蛋白酶大約相等。A_1、A_2、B、C等活性稍低於此兩種市販胰臟蛋白酶。對BAEE及TAME的活性測定得到相似的結果。

粗蛋白酶的最適pH是8等電點偏於酸性，最適溫度爲50°C差右在0～5°C南極蝦蛋白酶仍有5%活性。蛋白酶粗抽出渡在高溫不安定，在40°C以上保溫10分鐘後，活性卽顯著下降。但是粗蛋白酶在低溫或以硫酸銨飽和或以乾燥狀態保存，都具有很高的穩定性。粗蛋白酶溶液貯藏在5°C經過321天後，活性尚有64%，在－20°C及－30°C殘存活性均爲90%。凍結乾燥後貯藏在20°C、5°C及－20°C經過321天殘存活性分別爲84%、95%及98%。貯藏於硫銨飽和液中之蛋白酶，經過321天，其在－20°C者，活性幾乎沒有損失，而在5°C者仍有81%殘存活性。貯藏實驗獲得以下結論：蛋白酶溶液的最適貯藏條件是－20°C及－30°C，乾燥蛋白酶可以貯藏在20°C，蛋白酶於硫酸銨飽和液中，可以貯藏在5°C。

Freezing Preservation of Fresh Antarctic Krill (*Euphausia superba*)

Shann-Tzong JIANG*, Mao-Song CHEN** and Shyh-Shiuan CHANG**

ABSTRACT

It is well known that the frozen raw krill exudes a lot of drip and develops darkening discoloration during thawing. In order to reduce drip loss and prevent the development of darkening discoloration of frozen krill on thawing, some special pretreatments were employed and the following results were obtained.

For frozen-thawed Antarctic krill, pretreatments with the mixture of 3% NaCl and 1.0% sodium erythorbate or 0.2% polyphosphate-2DK (50% Na-polyphosphate, 50% Na-pyrophosphate) and 1.0% sodium erythorbate for 10 minutes at solution temperature below 5°C showed less drip exudation on thawing at both 5°C and 25°C than those treated otherwise and no darkening discoloration was observed until the samples were spoiled.

SUMMARY

Experiments were carried out to find some better pretreatments for preventing the development of darkening discoloration and reducing the drip loss from frozen krill. The following results were obtained.

1). When frozen raw krill was thawed at 5°C and 25°C of air temperature for 24 hours, about 20% and 33% of drip exuded and 30.0mg and 46.7mg of

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