Characteristics of Amylase and Protease of the Shrimp *Penaeus japonicus**¹

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(Received May 10, 1982)

The characteristics of the primary digestive enzymes of the shrimp *Penaeus japonicus* were studied in animals fed for 30 days on a compounded dry diet containing 60% protein.

Pepsin activity was not detected. A broad range of stable protease activity from pH 7.0-8.5 was found, indicating that perhaps more than one alkaline protease was detected by casein hydrolysis. Amylase activity showed a distinct activity optimum at pH 6.8. Optimum assay temperature for both amylase and protease was 40°C.

The effect of Ca²⁺ addition on relative amylase activity was opposite to that of protease. This may indicate that maximal expression of amylase activity requires a greater amount of calcium than that of protease activity. Sufficient amounts of calcium for maximal expression of amylase activity did not seem to be supplied by either environment or dietary sources.

GATES and TRAVIS¹⁾ purified a trypsin protease and later isolated carboxypeptidase A and B²⁾ from the hepatopancreas of *Penaeus setiferus*. DEVILLEZ and BUSCHLEN³⁾ in a survey of the tryptic digestive enzymes of crustacea, found trypsin activity in the gastric juice of the prawn *Pandalus platycerous*.

Generally, proteases of crustacea showed maximum activity in the range of pH 5.0 to 7.0.⁴⁾ However, DEVILLEZ⁵⁾ reported a pH optima of 7.5 to 8.5 for a tryptic protease of *Orconectes*, and ASAHARA⁶⁾ demonstrated that a protease from hepatopancreas of the shrimp *Trachypenaeus curvirostris* had a maximum activity at pH 8.5. Activity in the acidic range in decapod crustacea is rare and the existence of an enzyme with pepsin specificity has only recently been shown in *Machrobrachium rosenbergii*.⁷⁾

Basic research on the characteristics of the primary digestive enzymes of *Penaeus japonicus*, as indicated in the current study, is a prerequisite to understanding the digestive physiology of this commercially important species. Studies of this type should have an application in the develop-

ment of crustacean diets.

Materials and Methods

Source of Shrimp, Feeding Method, and Diet Composition

Penaeus japonicus, 3-4 g in size, were obtained from the Mitsui Norin shrimp farm (Kagoshima). They were maintained in covered 70 l plastic tanks fitted with a sand bed supported by a false bottom.⁸⁾ Prior to conducting the enzyme assays, the shrimp were fed a compounded dry diet, *ad libitum* during a four week period at water temperatures of 20-24°C. Table 1 presents the dietary components and proximate composition of the diet.

Tissue Extraction Procedure and Other Analysis

Amylase and protease activities in the hepatopancreas of shrimp were determined after the four week acclimation period. Animals were sacrificed at 10 a.m., approximately 16 h after the final feeding to avoid any changes in activity due to diurnal rhythm.¹¹⁾

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Table 1. Composition and proximate analysis of the compounded diet*¹

Ingredient	Percentage
Squid meal	42.3
Shrimp meal	20.0
Wheat gluten	10.0
Yeast* ²	15.0
NaH ₂ PO ₄ ·2H ₂ O	6.0
KCl	1.0
Vitamin mixture* ³	2.6
Cellulose powder	2.6
Cholesterol	0.5
Proximate composition* ⁴	
Crude protein	58.2
Crude fat	5.5
Ash	19.1
Carbohydrate	16.2
Moisture	10.2

*¹ The composition was modified from the basal diet of DESHIMARU and KUROKI⁽⁹⁾ and SHIGUENO *et al.*⁽¹⁰⁾

*² Dry powder of *Candida* sp.

*³ Thiamine-HCl, 5.0; Riboflavin, 16.4; Pyridoxine-HCl, 55.0; Nicotinic acid, 65.8; Ca-pantothenate, 24.7; Inositol, 328.8; Biotin, 0.5; Folic acid, 1.2; *p*-Aminobenzoic acid, 32.9; Choline-Cl, 657.7; α -Tocopherol, 1033; Menadione, 3.3; β -carotene, 3.3; Calciferol, 0.5; Cyanocobalamin, 0.05; Ascorbic acid, 370 mg.

*⁴ Mean values of triplicate analysis.

In the standard enzyme assay, hepatopancreas samples were removed washed with distilled water, blotted dry and weighed. The maximum amount of soluble protein, amylase and protease activities as related to the concentration of glycerol and NaCl in the extracting solution are given in Table 2. The 20% glycerol solution containing 0.2 M NaCl gave maximum amylase activity and was selected for all subsequent extractions. The samples were individually ground in centrifuge tubes at 0–4°C using a polytron tissue grinder (with 10 v/w of 20% glycerol solution containing

0.2 M NaCl). Extracts of the homogenates were centrifuged at 5,000 × g for 10 min to –4°C and were then used in the enzyme assays. The amount of soluble protein in the extracts were determined using a protein assay Test Kit (Bio Rad, Richmond, CA., USA). The standard curve was made using bovine plasma albumin (Bio Rad Co.), as a standard protein.

The proximate analysis of the compounded diet was performed as follows: Moisture content of the diet was determined by difference after the sample was dried for 24 h in an electric oven at 105°C. Crude fat was extracted with ethyl ether using the Soxhlet method and weighed gravimetrically. Crude protein was determined by the semi-micro Kjeldahl method, and carbohydrate was determined as glycogen by the method of CARROL *et al.*⁽¹²⁾ Ash was measured after dry ashing at 600°C for 16 h.

Amylase Assay

Amylase activity was determined using a Test Kit (Daiichi Kagaku Co., Tokyo) based on the RANDERKNECHT *et al.*⁽¹⁸⁾ method employing the amylase blue starch method. The substrate provided in the test kit was previously stained with Chibachron Blue F3GA (Ciba A. G. Switzerland) and made insoluble by the action of amylase, the amount of soluble, colored products were determined spectrophotometrically. The Hyland standard soluble multiple enzyme solutions (Hyland Co., USA) were used to determine a calibration curve for amylase.

In a typical assay, 0.1 ml of the extract as described above, was added to 4 ml of 0.2 M phosphate buffer (pH 6.8) and heated at 40°C for 5 min. The assay was initiated by the addition of the dyed starch substrate, the reaction mixture was completely mixed using a vortex mixer. After

Table 2. Protein solubility, and amylase and protease activities in *Penaeus japonicus*, as a function of the extract solution glycerol and NaCl content*

Extract solution Glycerol (%)	NaCl M	Mg protein-N per ml	Amylase			Protease		
			Specific activity	Total activity	(%)	Specific activity	Total activity	(%)
Distilled water		0.68	374.0 ± 11.0	479.0 ± 6.0	100	21.0 ± 1.0	828.0 ± 2.0	100
20	–0–	0.84	228.0 ± 25.0	478.0 ± 52.0	96	—	—	—
20	0.05	0.58	208.0 ± 5.0	294.0 ± 7.0	59	48.0 ± 1.0	119.0 ± 1.0	14
20	0.20	1.84	194.0 ± 11.0	881.0 ± 92.0	117	123.0 ± 1.0	306.0 ± 2.0	37
20	0.60	1.21	150.0 ± 3.0	442.0 ± 7.0	85	185.0 ± 2.0	430.0 ± 3.0	52
20	1.00	1.69	203.0 ± 5.0	228.0 ± 18.0	46	533.0 ± 2.0	1,133.0 ± 4.0	137

* Data given as mean ± S.D. of triplicate analysis. Hepatopancreas samples from size shrimp, 0.38 g mean weight were used in the extract solution. Amylase and protease activities given as described in the Materials and Methods section.

30 min incubation period the reaction was stopped by the addition of 1 ml of 0.1 N NaOH, and allowed to cool to room temperature. After the mixture was centrifuged at 3,000 rpm for 30 min, the absorbance of the supernatant was determined at 620 nm. Total amylase activity was recorded as IU/g/min, and apparent specific activity was IU/mg Protein-N/min.

Protease Assay

The assay for protease activity was a modification of the casein method of KUNITZ.¹⁴⁾ The substrate was made by dissolving 5 g casein of Hammarstein quality in 500 ml of 0.1 M phosphate buffer (pH 7.5) on a boiling water bath. The 1% casein was stored at 4°C. A 0.1 ml protein of hepatopancreatic homogenate, diluted to 10 v/w was added to 1.0 ml of 0.2 M phosphate buffer (pH 8.0) and heated to 40°C for 5 min. The assay was initiated by the addition of 1 ml of the substrate solution to the reaction mixture. After exactly 30 min incubation, 3 ml of 5% trichloroacetic acid solution was added to stop the reaction. The incubation mixture was cooled to room temperature, centrifuged at 3,000 rpm for 30 min, and filtered using No. 2 filter paper (Toyo Roshi Co., Tokyo). The amount of hydrolysis product in the filtrate was determined as tyrosine at 280 nm (Shimazu UV-201 spectrophotometer). The amount of enzyme liberation 1 μ M tyrosine per g tissue per min was taken as the total amount of proteolytic activity. Apparent specific activity was expressed as μ M tyrosine-liberated per mg soluble protein-N per min.

Effect of Metals and Chemicals on the Enzyme Activity

The effects of various metals and chemical reagents on the activities of both amylase and protease were tested. The enzyme solution (0.1 ml) and 1×10^{-8} M of various metallic ions and chemical reagents, dissolved in the appropriate 0.1 M phosphate buffer were incubated at 40°C for 1 h prior to the start of the enzyme assay. The experimental procedure in all other aspects was the same as described above.

Results and Discussion

Enzyme Activity as a Function of pH and Temperature

The influence of the assay solution pH on protease and amylase activities is shown in Figs. 1

Fig. 1. Activity of *Penaeus japonicus* protease as a function of pH. Buffers (0.2 M) used in assay solutions include: pH 2.0–3.5 Glycine-HCl; 3.0–7.0 Citrate-phosphate; 5.5–8.0 phosphate; 7.5–9.0 veronal; and 9.0–10.5 Carbonate-bicarbonate. Data is given as mean $\pm$ S.D. of triplicate assays.

Fig. 2. Activity of amylase from *Penaeus japonicus* hepatopancreas as a function of pH. Data given as mean $\pm$ S.D. of triplicate assays. Phosphate buffer 0.2 M, pH 5.5–8.0 were used.

and 2, respectively. The shrimp showed no distinct pepsin activity as found in *M. rosenbergii* by using a pepsin-specific substrate.⁷⁾ However, a broad range of activity may indicate that more than one alkaline protease was active in the hydrolysis of casein under the conditions of this study. Amylase activity, in contrast, showed a maximum at pH 6.8. Optimum temperature for both amylase and protease activities was 40°C (Fig. 3).

Effect of Metals and Chemicals

The results of these experiments are presented in Tables 3, 4 and 5 as percentages of relative

Fig. 3. The effect of temperature on the activity of protease and amylase in *Penaeus japonicus*. Amylase (○) and protease (●) are given as mean $\pm$ S.D. of triplicate assays.

Table 3. Effect of metal ions on the relative activities of amylase and protease in *Penaeus japonicus*

Percent amylase activity*1	Metal ions*2	Percent protease activity*2
100	No addition	100
94	LiSO ₄	101
81	NaCl	102
112	Na ₂ SO ₄	96
90	Na ₂ NO ₂	89
91	KCl	101
96	K ₂ SO ₄	100
98	MgSO ₄	100
104	CaCl ₂ (1×10^{-3})	89
116	CaCl ₂ (1×10^{-2})	73
94	BaCl ₂	96
89	MnCl ₂	90
85	FeSO ₄	112
93	Fe(NO ₃) ₂	96
91	Co(NO ₃) ₂	90
75	Ni(NO ₃) ₂	83
87	CuCl ₂	64
81	CuSO ₄	81
75	AgSO ₄	70
91	ZnSO ₄	98
98	Cd(NO ₃) ₂	73
3	HgCl ₂	12
104	SnSO ₄	93
85	Pb(NO ₃) ₂	94

*1 Amylase and protease activities are expressed as percentages relative to control values.

*2 Final concentration in the reaction solution was 1×10^{-3} M, unless otherwise noted.

activity to control values. Table 3 shows the effect of 18 metallic ions on protease and amylase activity. The effects of these metal ions on protease activity were somewhat different than those

Table 4. Effect of reducing agents and oxidants on relative amylase and protease activities

Percent amylase activity	Compound	Percent protease activity
100	No addition	100
86	L-Ascorbic acid at 1×10^{-3} M	99
90	at 1×10^{-2} M	87
87	L-Cystine	97
85	Glutathione	99
94	Hydroquinone	99
108	Na ₂ S ₂ O ₃	102
109	K ₄ Fe(CN) ₆	94
101	KCN	96
111	2-Mercaptoethanol	96

Table 5. Effect of metal chelating and SH-group binding agents on the relative amylase and protease activities

Percent amylase activity	Compound	Percent protease activity
100	No addition	100
75	<i>o</i> -Phenanthroline	—
83	<i>p</i> -Chloromercuric-benzoate	77
91	Iodoacetic acid	86
88	α - α' -Dipyridyl	—
84	Citric acid	82
89	8-Hydroxyquinoline	69
82	EDTA	89
121	Tartaric acid	100
118	Aniline	94

found for the king crab *Paralithodes cantschatica*¹⁵⁾ or the shrimp *Trachypenaeus curverstris*.⁶⁾ The only metallic ion which the shrimp *P. japonicus* appeared to require for protease activation, was Fe²⁺. However, protease activity was inhibited by Ag²⁺, Ca²⁺ (at 1×10^{-2} M), Cu²⁺ and Cd²⁺, and almost completely inhibited by Hg²⁺, which gave a relative activity of 12%.

The relative activity of amylase increased when two ions, Na₂SO₄ and CaCl₂ (particularly at a concentration of 10^{-2} M), were added to the assay solution. The effect of Ca²⁺ on relative amylase activity was the opposite of its effect on protease activity. This finding may indicate that adequate amounts of calcium were available for maximal expression of protease activity. However, maximal expression of amylase activity seems to require greater amounts of calcium than that of protease and may not have been met by dietary or environmental source. DESHIMARU and YONE¹⁶⁾

have shown that a diet devoid of dietary calcium gives the greatest percent weight gain and feed efficiency in *P. japonicus*. In a subsequent study on the absorption of $^{45}\text{Ca}^{2+}$, DESHIMARU *et al.*¹⁷⁾ postulated that the calcium requirement in shrimp could be satisfied by environmental sources when it was not present in the diet. In part, the results of the present study confirm that calcium is not necessary for an increase in relative protease activity. However, these studies^{16,17)} and the present study employed diets which contained relatively high levels of protein and a diminution in amylase activity due to a lack of calcium supplement probably had little effect on overall growth, percent weight gain, or feed efficiency.

In Tables 4 and 5, the effects of reducing agents, oxidants, metal chelating, and sulfhydryl group (SH) binding agents on amylase and protease activities are shown. The relative amylase activity was slightly increased by $\text{Na}_2\text{S}_2\text{O}_3$, $\text{K}_3\text{Fe}(\text{CN})_6$, 2-mercaptoethanol, tartaric acid, and aniline, and slightly inhibited by *o*-phenanthroline. The only increase in relative protease activity was demonstrated with $\text{Na}_2\text{S}_2\text{O}_3$. The finding that L-ascorbic acid decreased the activities of both amylase and protease, is in part supported by the results of ASAHARA,⁸⁾ which employed a similar extract procedure. This study reported that a 1×10^{-3} M addition of L-ascorbic acid decreased relative protease activity to 32% of control values.

The inhibition of protease activity by *p*-chloromercuribenzoate and iodoacetic acid indicates that the protease enzyme(s) probably contain SH-group(s), and the inhibition by several metal chelating substances, particularly 8-hydroxyquinoline may indicate that it requires metal ions for complete activation. However, there is little evidence that the addition of metal ions had a positive effect on protease activity.

Since the hepatopancreas is the major storage site for organic and inorganic reserves,^{4,18)} it seems likely that the metallic ion(s) required for optimum protease activity, with the exception of Fe^{2+} , may have been supplied from the environment. This is in agreement with the previous finding of DESHIMARU *et al.*^{16,17)} for calcium, in *penaeus japonicus*.

Acknowledgements

The authors are grateful to Dr. K. SHIGENO,

Director of the Kagoshima Prefecture Fisheries Experimental Station, for his cooperation in the use of facilities and to the Japanese Ministry of Education who provided financial support in the form of a Graduate Studies Fellowship under the International Exchange Visitor Program.

This study was also sponsored by NOAA Office of Sea Grant, Department of Commerce, under Grant No. NA 881AA-D-00073, and the University of Rhode Island Agricultural Experiment Station, Contribution No. 2075.

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