TRYPSIN-LIKE ENZYMES FROM TWO MORPHOTYPES OF THE 'LIVING FOSSIL' *TRIOPS* (CRUSTACEA: BRANCHIOPODA: NOTOSTRACA)

ALEJANDRO M. MAEDA-MARTÍNEZ, VERÓNICA OBREGÓN-BARBOZA,
M. ANGELES NAVARRETE-DEL TORO, HORTENCIA OBREGÓN-BARBOZA
and FERNANDO L. GARCÍA-CARREÑO

Reprinted From: Comparative Biochemistry and Physiology
Volume 126 Number 3

ELSEVIER

Comparative Biochemistry and Physiology Part B 126 (2000) 317–323

CBP

www.elsevier.com/locate/cbpb

Trypsin-like enzymes from two morphotypes of the 'living fossil' *Triops* (Crustacea: Branchiopoda: Notostraca)

Alejandro M. Maeda-Martínez *, Verónica Obregón-Barboza, M. Angeles Navarrete-Del Toro, Hortencia Obregón-Barboza, Fernando L. García-Carreño

Centro de Investigaciones Biológicas del Noroeste, S.C., Apdo. Postal 128, La Paz, Baja California Sur, Mexico

Received 23 September 1999; received in revised form 10 February 2000; accepted 25 February 2000

Abstract

Trypsin-like enzymes from two morphotypes (here called short and long) of the 'living fossil' *Triops* of Baja California Sur, México were studied. Adults of both morphotypes were obtained from outdoor static cultures using dry soil from the natural habitats as a source of cysts and culture substrate. Individual and pooled extracts were made from dissected digestive tubes. The effect of pH and temperature on the trypsin activity was studied using *N*-alpha-benzoyl-DL-Arg-*p*-nitroanilide (BAPNA) as substrate. The highest proteolytic activity was found at the same pH with extracts of both morphotypes. At this pH, there was greater proteolytic activity at a lower temperature with the short morphotype extract than with the long morphotype extract. Substrate-SDS-PAGE zymograms showed bands of activity. Short morphotype extracts produced six bands; five of them were serine proteases of which three were trypsin-like enzymes. Long morphotype extracts revealed eight bands; six of them were serine proteases of which three were trypsin-like enzymes.

Keywords: Crustacea; Digestive enzymes; Serine proteases; Substrate-SDS-PAGE; *Triops*; Trypsin

1. Introduction

The branchiopod genus *Triops* of the order Notostraca (tadpole shrimp) occurs in all continents except Antarctica. Its natural habitats are ephemeral freshwater bodies that are characterized by their extreme physical and chemical conditions. The survival and prevalence in these unstable environments can be explained by a number of special adaptive traits, e.g. during the dry phase of the habitat survival is guaranteed in part by the production of dormant eggs (so called cysts) that withstand dessication until next rainfall. *Triops* is considered a 'living fossil' because it has undergone minimal morphological change over 180 million years. The extant species *Triops cancriformis* can be considered conspecific with the fossil form found from Triassic material (Longhurst, 1955).

Notostracans are omnivorous and predominantly benthic; they feed on detritus or on live or dead organisms (Martin, 1992). Some forms of *Triops* have been reported as a pest in rice fields of California, USA, and Japan (Grigarick et al., 1961). Although in the past, farmers attempted to eliminate this shrimp, more recently it has been

* Corresponding author. Tel.: +52-112-53633; fax: +52-112-54715.

E-mail address: almaeda@cibnor.mx (A.M. Maeda-Martínez)

0305-0491/00/$ - see front matter
PII: S0305-0491(00)00183-8

proposed they be used to control weeds in the same rice fields (Takahashi and Gohda, 1981).

Linder (1952) proposed that all *Triops* species described from the North American continent (including Mexico) were synonyms of *Triops longicaudatus*. However, Sassaman et al. (1997) recently demonstrated by electrophoretic and morphological analyses that this species is a mixture of at least two reproductively isolated species. Two *Triops* morphotypes in the state of Baja California Sur, Mexico occur exhibiting some interesting biological characteristics such as a rapid growth, early maturation (6 days), and uniparental reproduction via cysts (pers. obs.). These features make these crustaceans attractive for aquaculture. However, the optimal use and culture of an organism require knowledge of basic aspects such as the nutrition of the species. Vital processes, development and reproduction, are based on a highly effective mechanism of regulation to maintain an adequate metabolism under extreme conditions. Digestive enzymes are basic elements of this system because these act as mediators between the intake of food and its assimilation (Dittrich, 1992).

Available literature on the digestive physiology of notostracans is nil. Our aim is to contribute to the knowledge of digestive proteases of *Triops* through a comparative study of the trypsin activity of the morphotypes from Baja California Sur, to eventually arrive at some conclusions about their nutrition physiology. Thus, the objectives were to characterize the effect of pH and temperature on the trypsin activity, and to determine the relative molecular mass of trypsin-like enzymes from extracts of these organisms.

2. Material and methods

Specimens of both *Triops* forms, called here short and long morphotypes, were obtained from outdoor static cultures established at Centro de Investigaciones Biológicas del Noroeste, S.C. (CIBNOR), La Paz, Baja California Sur, México. The cultures were made in 1200 l tanks by adding 20 kg of dry soil containing *Triops* cysts from natural habitats. The tanks were filled with tap water (total dissolved solids = 0.6 g/l) on 29 August 1998. Soil samples were from the following localities. The short morphotype was taken from an ephemeral pond, at the junction La Paz-San Juán de la Costa, Federal highway No. 1, La Paz-Cd. Constitución, Baja California Sur: 24°07′12″ N, 110°26′15″ W. The long morphotype was obtained from an ephemeral pond at km 75.5, Federal highway No. 1, Todos Santos-Cabo San Lucas, Ejido Elías Calles, Baja California Sur: 23°14′05″ N, 110°09′01″ W. Identification of the morphotypes was made according to Sassaman et al. (1997).

For each morphotype, one pooled and one individual extract were obtained from adults (individuals with eggs in ovisacs) of no more than 20 days old. Short-morphotype pooled extract was obtained from 15 animals. The mean and standard deviation of the carapace length and live weight of these animals were 14.88 ± 0.57 mm and 0.43 ± 0.04 g. Long-morphotype pooled extract was obtained from 14 animals. The mean and standard deviation of the carapace length and live weight of these animals were 19.89 ± 0.54 mm and 1.15 ± 0.16 g. The short-morphotype individual extract was obtained from a shrimp of 15.5 mm carapace length and 0.46 g live weight. The long-morphotype individual extract was obtained from a shrimp of 20.5 mm carapace length and 1.85 g live weight. Individual extracts were used only in the substrate-SDS-PAGE zymograms to confirm the pattern of bands of activity exhibited by the pooled extracts (Fig. 3). Extracts were made from dissected digestive tubes according to the following protocol: the specimens were washed with distilled water and dried on paper. Using a pair of scissors, a section of the anterior right part of carapace was cut and the blood was drained for 2 min. Both telson and head (at mandibular region) were cut tranversally. From the anterior part of the animal and with the aid of a pincers, the digestive tube was removed. In an ice bath, homogenates were made using a Potter homogenizer with 50 mM Tris–HCl, 20 mM $CaCl_2$, pH 7.5 in proportion of 1:2 w/v (digestive tube weight: buffer). Homogenates were centrifuged at $10\,000 \times g$ during 20 min at 4°C. Supernatants were divided into aliquots of 30 μl and stored at −30°C.

The soluble protein content of the extracts was determined according to Bradford (1976) using bovine albumin as a standard (1 mg/ml). The absorbance was determined with a UV-visible spectrophotometer (Beckman DU 640) using the Bradford reagent as a blank. The assays were done in triplicate. The effect of pH and tempera-

ture on the trypsin activity was studied using *N*-α-benzoyl-DL-Arg-*p*-nitroanilide (BAPNA) as the substrate (Erlanger et al., 1961; García-Carreño and Haard, 1993). For pH assays, BAPNA was prepared at a concentration of 1 mM and dissolved in 1 ml of dimethyl sulfoxide (DMSO). The mixture was homogenized in a vortex mixer for 10 min, and made to 15 ml with universal buffer (Stauffer, 1989) prepared at pH 6, 7, 8, and 9. To 1 ml of substrate solution, 5 μl of crude extract was added and the increase of absorbance

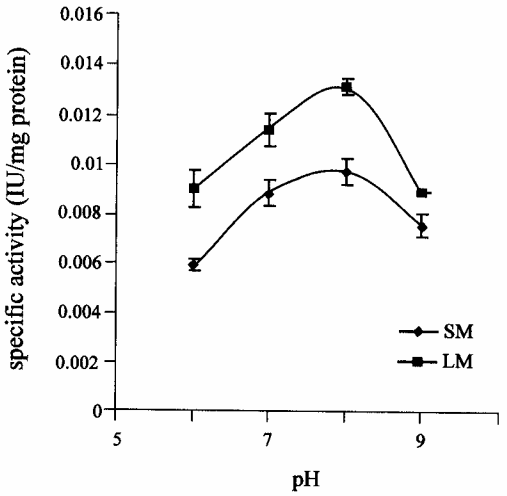

Fig. 1. Effect of pH on the trypsin activity of enzyme extracts of both short (SM) and long (LM) morphotypes of *Triops*. The values are the means with the standard deviation.

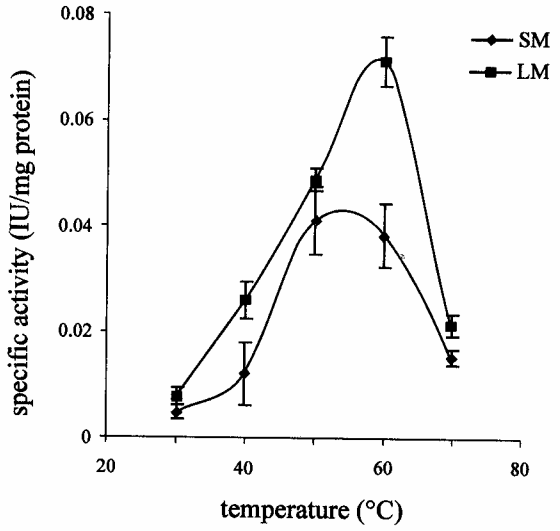

Fig. 2. Effect of temperature on the trypsin activity of enzyme extracts of both short (SM) and long (LM) morphotypes of *Triops*. The values are the means with the standard deviation.

at 410 nm was recorded over 10 min at 28°C. The assays were done in triplicate. The substrate solution was used as the control. The specific activity was determined in International Units (IU) according to the formula: specific activity (IU) = Abs_{410}/min × 1000 × vol/8800 × mg protein in assay; where 8800 is the coefficient of molar extinction of *p*-nitroaniline (Dimes et al., 1993). One unit of activity is expressed as 1 μmol of *p*-nitroaniline released/min. The temperatures studied were 30, 40, 50, 60, and 70°C. BAPNA was prepared at a concentration of 1 mM and dissolved in 1 ml of DMSO. The mixture was homogenized in a vortex mixer for 10 min, and made to 20 ml with 50 mM Tris–HCl, 20 mM $CaCl_2$, pH 8.0. This solution was warmed before the assay. The buffer pH was adjusted at the assay temperature. The enzyme solution was prepared with 5 μl of crude extract and 95 μl of 50 mM Tris–HCl, 20 mM $CaCl_2$, pH 8.0. The reaction was done by adding 1000 μl of substrate solution and the reaction mixture incubated for 10 min. The reaction was stopped by adding 250 μl of 40% acetic acid. Before adding the substrate solution, acetic acid was added to the controls. The absorbance was determined at 410 nm, using distilled water as the blank. The assays were done in triplicate in a water bath (Precision Scientific 180). The effect of both pH and temperature on the specific activity (Figs. 1 and 2) was statistically tested using a multivariate analysis of variance (MANOVA) (STATISTICA 5.0). To evaluate differences of means between the two morphotypes a Student's *t*-test was done for each treatment. Electrophoretic separation of proteins was done according to Laemmli (1970) in twin gels of 12% polyacrylamide (8 × 10 × 1.5 mm), using the vertical system Mini-PROTEAN II (Bio-Rad, 1970). Crude enzyme extracts were diluted 1:2 in 0.125 mM Tris–HCl, pH 6.8, 4% sodium dodecyl sulfate (SDS), 0.02% bromophenol blue, and 20% glycerol. From individual extracts, 20 μg of total protein were used, and from pooled extracts a solution with 10 mU was used without boiling. The electrophoresis was made at constant current of 15 mA per plate at 4°C. The profile of bands of proteases were revealed according to García-Carreño et al. (1993). After electrophoresis, the gels were washed in distilled water and incubated for 30 min at 5°C in 40 ml of 3% casein prepared in 50 mM Tris–HCl, pH 7.5. The gels were incubated again in fresh casein solution for 90 min at

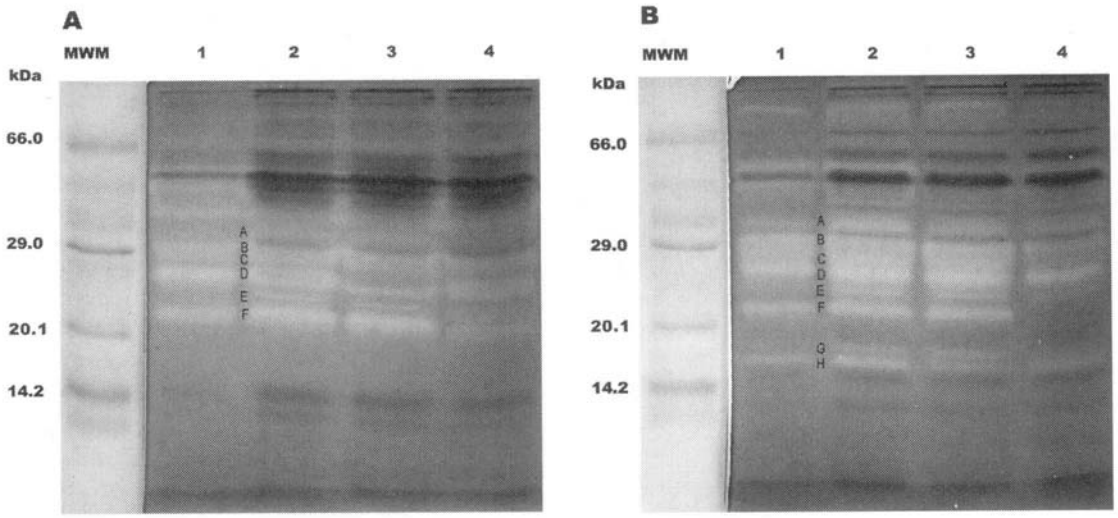

Fig. 3. Substrate-SDS-PAGE zymograms. A: Short morphotype extracts. B: Long morphotype extracts. MWM, molecular mass markers (Sigma) in kDa: Bovine albumin (BSA) 66.0; ovalbumin 45.0; glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle 36.0; carbonic anhydrase from bovine erythrocytes 29.0; trypsinogen from bovine pancreas 24.0; trypsin inhibitor from soybean 20.1; α-lactalbumin from bovine milk 14.2; aprotinin from bovine lung 6.5. Lane 1, individual extract. Lane 2, pooled extract. Lane 3, pooled extract incubated 1 h with 10 mM TLCK. Lane 4, pooled extract incubated 1 h with 100 mM PMSF. Letters inside the zymograms between lanes 1 and 2 represent the bands of activity referred to the text, and Table 1.

25°C with constant agitation (50 rpm). To determine the proteases with trypsin activity in the zymograms, inhibition assays in gels were done. Before electrophoresis, 30 μl aliquots of crude extracts were incubated for 1 h at 25°C with 3 μl of 100 mM phenylmethanesulfonyl fluoride (PMSF) and 10 mM tosyl-Lys chloromethyl ketone (TLCK) (García-Carreño and Haard, 1993). The molecular mass markers used in the zymograms are indicated in the legend of Fig. 3.

3. Results

The effect of pH on the trypsin activity of extracts of both morphotypes was significant ($P < 0.01$) (Fig. 1). The highest proteolytic activity was at pH 8 with extracts of both morphotypes. In all pH values tested, the long morphotype extract had a significant higher activity than the short morphotype extract ($P < 0.05$). The effect of temperature on the specific activity of extracts of both morphotypes was significant ($P < 0.001$) (Fig. 2). With short morphotype extract, there was major proteolytic activity between 50 and 60°C, whereas with the long morphotype extract, the major activity was at 60°C. At this temperature the long morphotype extract exhibited a significantly higher activity than the short morphotype extract ($P < 0.01$). Also, at 40 and 70°C, the long morphotype extract showed a significantly higher activity than the short morphotype extract ($P < 0.05$).

Zymograms from both morphotypes had clear bands of activity within the range of 32–14 kDa (Fig. 3). Short morphotype extracts produced six bands of activity (Fig. 3A); five of them were serine proteases (inhibition with PMSF) with relative molecular masses of 31.6 (A), 27.5 (C), 24.5 (D), 22.7 (E), and 20.5 (F) kDa. Bands A, D, and E are trypsin enzymes (inhibition with TLCK). Band B, a protease of 29.3 kDa was not affected with the inhibitors assayed. Long morphotype extracts revealed a proteolytic pattern of eight bands (Fig. 3B); six of them were serine proteases with relative molecular masses of 30.8 (A), 26.5 (C), 22.7 (E), 20.5 (F), 16.7 (G), and 15.5 (H) kDa. Bands A, G, and H are trypsin enzymes. Bands B and D of 28.5 and 23.9 kDa were not affected with the inhibitors tested.

4. Discussion

In general, trypsins show a pH optimum between 7 and 9 (Eggerer, 1984). The trypsin activity of the extracts of both morphotypes had major proteolytic activity at pH 8 (Fig. 1). This value

agrees with those reported for other crustaceans. Olalla et al. (1978) determined the influence of pH on two proteases of *Artemia* larvae using BAPNA as substrate and found that both enzymes had a flat peak of activity between 7.5 and 10.0 pH. Pan et al. (1991), using benzoyl-L-arginine-ethyl-ester (BAEE) as substrate, found that the major trypsin activity of extracts of *Artemia* of 70 h age was at pH between 7 and 8 with a peak at 7.5. Gates and Travis (1969) found a trypsin-like enzyme from the midgut gland of the shrimp *Penaeus setiferus* with a pH optimum between 7.0 and 9.5 using Bz-L-ArgEt as substrate. Zwilling and Neurath (1981) reported crayfish trypsin with a pH optimum between 7 and 8. Jiang et al. (1991) found three enzymes from digestive extracts of *Penaeus monodon* that showed a highest hydrolysis of *p*-toluenesulfonyl-L-arginine methyl ester (TAME) at pH 8.0 in two of them and at pH 7.0 in the third. Using TAME, Dionysius et al. (1993) reported a trypsin enzyme from midgut extracts of the sand crab *Portunus pelagicus* with a maximum proteolytic activity at pH 8.0.

For the influence of temperature on the trypsin activity in *Triops*, we found that at pH 8.0 the short morphotype extract had major proteolytic activity between 50 and 60°C, whereas the long morphotype extract showed major activity at 60°C (Fig. 2). Similar values have been reported as the optimum temperature for trypsins in other crustaceans. Jiang et al. (1991) found that two trypsin enzymes from extracts of *Penaeus monodon* at pH 8 exhibited a maximum caseinolytic activity at 65°C, whereas a third trypsin showed its highest activity at 55°C at the same pH. Dionysius et al. (1993) reported a trypsin enzyme from the midgut of the sand crab *Portunus pelagicus* with a maximum activity on azocasein at 60°C at pH 7.5. Dittrich (1992), using L-benzoyl-arginine-*p*-nitroanilide (L-BAPA) as substrate at pH 8.3, found that digestive extracts of *Clibanarius striolatus* (53°C), *Uca urillei* (52°C), *Pagurus bernhardus* (48°C), *Euphasia superba* (53°C), *Glyptonotus antarticus* (49°C), and *Calanus acutus* (42°C) had their highest activity at the temperatures noted.

Trypsins have been reported with relative molecular masses as high as 50.1 kDa from *Penaeus monodon* (Jiang et al., 1991), and as low as 13.0 kDa from porcine alpha trypsin (Walker and Keil, 1973). In this study, extracts of both *Triops* morphotypes had active bands within the range of 32–14 kDa (Fig. 3). Comparing the zymograms, we found the following differences: (i) the short morphotype extracts had six bands of proteolytic activity, whereas the long morphotype extracts had eight. The two additional bands (G and H) from the long morphotype extracts correspond to relative molecular masses of 16.7 and 15.5 kDa; (ii) although the remaining six bands have similar relative molecular masses between the morphotypes, their response to the inhibitors PMSF and TLCK was different (Fig. 3, Table 1). Band D in the short morphotype was inhibited with both chemicals, whereas the corresponding band in the large morphotype seems not affected. Band E in the short morphotype showed inhibition with both chemicals, whereas the corresponding band

Table 1
Relative molecular mass (Mr in kDa) and effect of inhibitors PMSF and TLCK on the proteolytic activity of digestive enzymes of both short and long morphotypes[a]

Band	Short morphotype			Long morphotype		
	Mr	PMSF	TLCK	Mr	PMSF	TLCK
A	31.6	+[b]	+[b]	30.8	+[b]	+[b]
B	29.3	−[c]	−[c]	28.5	−[c]	−[c]
C	27.5	+[b]	−[c]	26.5	+[b]	−[c]
D	24.5	+[b]	+[b]	23.9	−[c]	−[c]
E	22.7	+[b]	+[b]	22.7	+[b]	−[c]
F	20.5	+[b]	−[c]	20.5	+[b]	−[c]
G				16.7	+[b]	−[c]
H				15.5	+[b]	+[b]

[a] Data from zymograms of Fig. 3.
[b] +, effect of inhibition.
[c] −, no effect.

in the large morphotype had a clear inhibition only with PMSF. Zwilling and Neurath (1981) reported that crayfish trypsin occurs in multiple forms, where the number and electrophoretic mobility varies from one species to another; *Astacus fluviatilis*, for instance, has three electrophoretic bands of distinct trypsin specificity, whereas *Astacus leptodactylus* has only two. It is known that if one protease is inhibited by TLCK, it is not necessarily affected by the general inhibitor of serine proteases, PMSF. Sillero et al. (1980), separated four alkaline proteases (called A, B, C, and D) from larval extracts of both bisexual and parthenogenetic populations of *Artemia*. These proteases were tested with different substrates and inhibitors and showed that protease A was inhibited by PMSF but not by TLCK, and the enzymes B and C were inhibited by TLCK but not by PMSF. These workers concluded enzymes B and C are trypsin, and enzyme A is a chymotrypsin.

Digestive proteinases are important in nutrition physiology because they are responsible for the digestion of food protein, yielding amino acids used for tissue construction and hence growth. The long morphotype had higher proteinase activity than the short one. The long morphotype also exhibited a higher growth rate than the short morphotype when cultured under identical conditions (data to be published elsewhere). A possible correlation between the protein digestibility and growth rate is under study. The two *Triops* morphotypes studied here are indeed distinct biological entities, with differences not only in their morphology but also in their digestive physiology. Their biochemical differences found in the present study can be seen as additional evidence that these morphotypes represent two different species as proposed by Sassaman et al. (1997). Assuming that the *Triops* morphotypes are different species, the biochemical differences could be explained by divergent evolution of the serine proteases in these organisms.

Acknowledgements

We thank Dr E. Glazier for editing the English language text, Dr G. Murugan for helping in the statistical analyses, and G. Hernández-García for his assistance in the preparation of figures.

References

Bio-Rad, 1970. Protein II Slab Cell. Instruction Manual.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of dye binding. Anal. Biochem. 72, 248–254.

Dimes, L.E., García-Carreño, F.L., Haard, N.F., 1993. Estimation of protein digestibility. III. Studies on the digestives enzymes from the pyloric ceca of rainbow trout and salmon. Comp. Biochem. Physiol. 109A, 349–360.

Dionysius, D.A., Hoek, K.S., Milne, J.M., Slattery, S.L., 1993. Trypsin-like enzyme from sand crab (*Portunus pelagicus*): purification and characterization. J. Food Sci. 58, 780–792.

Dittrich, B.U., 1992. Life under extreme conditions: aspects of evolutionary adaptations to temperature in crustacean proteases. Polar Biol. 12, 269–274.

Eggerer, J., 1984. Hysteretic behaviour of citrate synthase: site-directed limited proteolysis. Eur. J. Biochem. 143, 205–212.

Erlanger, B.F., Kokowsky, N., Cohen, W., 1961. The preparation and properties of two new chromogenic substrates of trypsin. Arch. Biochem. Biophys. 95, 271–278.

García-Carreño, F.L., Haard, N.F., 1993. Characterization of proteinase classes in langostilla (*Pleuroncodes planipes*) and crayfish (*Pacifastacus astacus*) extracts. J. Food Biochem. 17, 97–113.

García-Carreño, F.L., Dimes, L.E., Haard, N.F., 1993. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceus proteinase inhibitors. Anal. Biochem. 214, 65–69.

Gates, B., Travis, J., 1969. Isolation and comparative properties of shrimp trypsin. Biochemistry 8, 4483–4489.

Grigarick, A.A., Lange, W.H., Finfrock, D.C., 1961. Control of the tadpole shrimp, *Triops longicaudatus*, in California rice field. J. Econ. Entomol. 54, 36–40.

Jiang, S.T., Moody, M., Chen, H.C., 1991. Purification and characterization of proteases from digestive tract of grass shrimp (*Penaeus monodon*). J. Food Sci. 56, 322–326.

Laemmli, U., 1970. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. Nature 277, 680–685.

Linder, F., 1952. Contributions to the morphology and taxonomy of the Branchiopoda Notostraca, with special reference to the North American species. Proc. US Nat. Mus. 102, 1–69.

Longhurst, A.R., 1955. A review of the Notostraca. Bull. Br. Mus. Nat. Hist. (Zool.) 3, 1–57.

Martin, J.W., 1992. Branchiopoda. In: Harrison, F.W., Humes, A.G. (Eds.), Crustacea. In: Microscopic Anatomy of Invertebrates, vol. 9. Wiley-Liss, New York, NY, pp. 25–224.

Olalla, A., Osuna, C., Sebastian, J., Sillero, A., Sillero, M.A.G., 1978. Purification and properties of three proteases from the larvae of the brine shrimp *Artemia salina*. Biochim. Biophys. Acta 523, 181–190.

Pan, B.S., Lan, C.C., Hung, T.Y., 1991. Changes in composition and proteolytic enzyme activities of *Artemia* during early development. Comp. Biochem. Physiol. 100A, 725–730.

Sassaman, C., Simovich, M.A., Fugate, M., 1997. Reproductive isolation and genetic differentiation in North American species of *Triops* (Crustacea: Branchiopoda: Notostraca). Hydrobiologia 359, 125–147.

Sillero, M.A.G., Burillo, S.L., Domínguez, E., et al., 1980. Multiple proteolytic enzymes in *Artemia*. In: Persoone, G., Sorgeloos, P, Roels, O., Jaspers, E. (Eds.), Physiology, Biochemistry, Molecular Biology. In: The Brine Shrimp *Artemia*, vol. 2. Universa Press, Wetteren, Belgium, pp. 345–354.

Stauffer, C., 1989. Enzyme Assay for Food Scientist. Avi Press, New York, NY.

Takahashi, F., Gohda, M., 1981. The type of egg production curve with respect to population preservation: ecological studies of tadpole shrimps (Branchiopoda: Triopsidae) for the biological control of weeds in rice paddies. Verh. Int. Ver. Limnol. 21, 1531–1540.

Walker, J.E., Keil, B., 1973. Purification and characterisation of different active forms of pork trypsin. Eur. J. Biochem. 32, 486–491.

Zwilling, R., Neurath, H., 1981. Invertebrate proteases. Method. Enzymol. 80, 633–664.