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PURIFICATION AND PROPERTIES OF THREE PROTEASES FROM THE LARVAE OF THE BRINE SHRIMP *ARTEMIA SALINA*

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Summary

Three proteases, termed A, B and C, have been characterized and partially purified from *Artemia salina* larvae. Enzyme A is active on benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester (K_m = 53 μM, determined at pH 8 and 37°C) but not on α-*N*-benzoyl-DL-arginine *p*-nitroanilide and is strongly inhibited by μM concentrations of phenylmethylsulfonylfluoride. Enzymes B and C are active on α-*N*-benzoyl-L-arginine *p*-nitroanilide (K_m = 20 and 11 μM, respectively) but not on benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester and B, but not C, is inhibited by mM concentrations of phenylmethylsulfonylfluoride. Enzymes A, B and C are optimally active at alkaline pH values, do not require either metal ions or -SH groups for their catalytic activity and have molecular weights of 38 000, 33 000 and 34 000, respectively. After heating for 5 min at pH 7.5 and in the presence of 0.7 M KCl half inactivation of proteases A, B and C was attained at 60, 52 and 45°C, respectively.

Introduction

Our work in the last years has been centered on the study of the development of *Artemia salina*, with special interest for the metabolism of nucleotides [1] and yolk platelets [2,3]. The content of yolk platelets of the cysted gastrula decreases drastically during the first 2 days of development [2]. The biochemical analysis of this phenomenon, together with the general role that proteases play during embryonic development, led us to the study of proteases in *Artemia*. The proteolytic activity of the dormant cysts is very low and increases in the first 2 days of development; during that time, and using proteolytic substrates, four activities have been detected termed A, B, C and D and

their course of appearance investigated [4]. Concerning the physiological role of these enzymes, it is known that, in vitro, protease B has a lytic action on *Artemia* yolk platelets [2,3] and RNA polymerase I [5]. Whether these proteases are from digestive or intracellular origin is not known at present.

The object of this report is to describe the purification and properties of three (A, B and C) of the four activities previously described.

Materials and Methods

Growth conditions of Artemia cysts

Artemia eggs were from Longlife Fish Products, Ontario, Canada. All operations were conducted at 0—4°C. Immediately before use, the eggs were resuspended in cold distilled water and washed several times, followed by treatment with cold 1% NaClO for 3 min. After decantation, the eggs were thoroughly washed with glass distilled water to remove residual NaClO. The washed eggs were further treated with 1% commercial tinture of merthiolate (Lilly) for 5 min followed by 3 washings; the eggs were incubated in the medium described by Dutrieu [6], in which KBr was replaced by NaBr. The components of this medium were (per litre): 40 g NaCl, 5 g $MgCl_2$, 2.5 g Na_2SO_4, 0.6 g KCl, 1 g $CaCl_2$, 0.2 g $NaHCO_3$, 0.1 g NaBr, 0.4 g H_3BO_3. *Artemia* cysts corresponding to 5 g dry eggs were incubated with 1.5 l of the above sterile medium supplemented with 30 U/ml penicillin and 0.1 mg/ml streptomycin. The flasks were stirred at approx. 175 rev./min at 30°C. In these conditions hatching occurs at about 18 h. In order to get certain degree of synchrony, emerging naupliae were collected taking advantage of their positive phototactism or using a decantation funnel [2]. This homogeneous population was further incubated in the same medium.

Enzymatic assays

The proteolytic activities present in *Artemia* extracts were measured with different substrates as shown below. Except when indicated, reactions were carried out in a final volume of 1 ml at 37°C. The enzymatic rates were corrected for the spontaneous hydrolysis of the substrates under identical experimental conditions. One unit is the amount of enzyme able to transform 1 μmol of substrate per min at 37°C.

Activity on benzyloxycarbonyl-L-leucine p-nitrophenyl ester

The reaction mixture contained 50 mM Tris · HCl (pH 8)/10% methanol/ 0.05 mM benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester and enzyme. For Km determination of protease A, initial velocities were determined in the presence of various (10, 15, 20, 30, 41 and 51 μM) substrate concentrations. Increase in absorbance at 400 nm was followed in a spectrophotometer. The molar extinction coefficient for *p*-nitrophenol is 18 000 A_{400} units. Rate measurements at 400 nm, when performed at pH values other than 8.0, were corrected for the pH dependence of the molar extinction coefficient for *p*-nitrophenol [7]. The stock solution of the substrate was prepared by dissolving it in dimethylsulfoxide at a final concentration of 2.5 mM.

Activity on α-N-benzoyl-DL-arginine p-nitroanilide

The reaction mixture contained 50 mM Tris · HCl (pH 7.5)/0.4 mM α-*N*-benzoyl-DL-arginine *p*-nitroanilide and enzyme. For K_m determinations of proteases B and C initial velocities were determined at various (7, 9, 11, 14, 23, 45, 90 and 400 μM) substrate concentrations of the L-isomer. Dimethylsulfoxide was adjusted to 1% in all cuvettes. Increase in absorbance was followed at 410 nm. The molar extinction coefficient for *p*-nitroaniline is 8800 A_{410} units. A stock solution of α-*N*-benzoyl-DL-arginine *p*-nitroanilide, at a final concentration of 1 mM, was prepared in 50 mM Tris · HCl (pH 7.5)/1% dimethylsulfoxide. Care was taken that all the substrate was dissolved in dimethylsulfoxide prior to the addition of the buffer [8].

Activity on (p-toluenesulfonyl)-L-arginine methyl ester

The assay procedure employed is exactly analogous to that described in ref. 9. The reaction mixture contained 40 mM Tris · HCl (pH 8.1)/10 mM $CaCl_2$/1 mM (*p*-toluenesulfonyl)-L-arginine methyl ester and enzyme. The change in absorbance was measured at 247 nm.

Activity on denatured casein

The reaction mixture contained 50 mM Tris · HCl (pH 7.5)/1% casein and enzyme. The incubation was done at 37°C and the reaction was stopped by adding 1 ml 10% trichloroacetic acid. After centrifugation, absorbance at 280 nm was determined in the supernatant. Casein solution was prepared as described by Arnon [8].

Activity on azocasein

The substrate solution was prepared by dissolving 0.62 g azocasein in 12.5 ml 2% $NaHCO_3$ at 60°C. The pH was adjusted to 7.2 with HCl and the volume made up to 50 ml with water. The reaction mixture contained (in a final volume of 0.5 ml) 0.3 ml azocasein solution and enzyme. The incubation was done at 37°C and the reaction was stopped by addition of 2 ml 5% trichloroacetic acid. The insoluble protein was removed by centrifugation and after addition of 0.5 ml 2.5 M NaOH to the supernatant, the absorbance was measured at 428 nm [10].

Other materials

The following compounds were obtained from Sigma Chemical Co.: α-*N*-benzoyl-DL-arginine *p*-nitroanilide; benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester; phenylmethylsulfonylfluoride and (*p*-toluenesulfonyl)-L-arginine methyl ester. Azocasein was from Calbiochem, casein from Kahlbaum, DEAE-SH-cellulose (0.85 meq/g) from Serva. Chymotrypsinogen, cytochrome c, myoglobin and ovalbumin used as molecular weight markers were from Schwarz/Mann. All other chemicals were of analytical grade.

Results and Discussion

Purification of enzymes

Proteases A, B and C were followed during the purification procedure

through their activity on benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester and α-*N*-benzoyl-DL-arginine *p*-nitroanilide, respectively. Operations were carried out at approx. 4°C.

Step 1: 150 000 × g supernatant. The larvae (18.4 g wet weight) obtained after incubation of 40 g eggs during 60 h were collected by filtration, washed with distilled water and hand-homogenized in a Kontes Duall Grinder with 20 ml 0.035 M Tris · HCl (pH 7.7)/0.07 M KCl/0.009 M $MgCl_2$/0.25 M sucrose. The homogenate was centrifuged at 27 000 ×*g* for 15 min and the resulting supernatant was further centrifuged at 150 000 ×*g* for 60 min. The supernatant presented activity on benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester and α-*N*-benzoyl-DL-arginine *p*-nitroanilide.

Step 2: precipitation with $(NH_4)_2SO_4$. To the solution from the previous step. 51.6 g of solid $(NH_4)_2SO_4$/100 ml (0.8 saturation) were added. After stirring for 30 min, the suspension was centrifuged at 27 000 ×*g* for 15 min and the supernatant was discarded. The precipitate was resuspended in 8.7 ml 20 mM Tris · HCl (pH 7.5).

Step 3: chromatography on Sephadex G-100. The solution from the previous step was applied to a Sephadex G-100 column (2.6 × 119 cm) equilibrated with 20 mM Tris · HCl (pH 7.5) and eluted with the same buffer. Fractions of 8.2 ml were collected. The elution profile of the activities on benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester and α-*N*-benzoyl-DL-arginine *p*-nitroanilide were coincident in great extent. Fractions 27—45, embracing both activities, were pooled.

Step 4: chromatography on DEAE-SH-cellulose. The pooled fractions from the previous step were applied to a column (1 × 20 cm) of DEAE-SH-cellulose previously equilibrated with 20 mM Tris · HCl (pH 7.5). The column was washed with 0.2 M KCl/20 mM Tris · HCl (pH 7.5) until the absorbance at 280 nm of the effluent was near zero. The volume of the fractions 1—49 was of 6.5 ml. The proteolytic activities were eluted with 200 ml of a linear gradient from 0.2—1.0 M KCl in 20 mM Tris · HCl, pH 7.5. Fractions of 1.6 ml were collected. At this step, two proteolytic activities on α-*N*-benzoyl-DL-arginine *p*-nitroanilide (B and C) are clearly separated (Fig. 1); their maximum activities correspond to fractions 88 and 108, respectively. The activity on benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester, corresponding to protease A chromatographs at about the same position as protease B, although the fraction with maximum activity (87) is slightly displaced to the left in relation to peak B. In one experiment not shown the proteolytic activity of the DEAE-SH-cellulose fractions was also assayed using casein as substrate. In that case a new peak of proteolytic activity was clearly observed between peaks B and C. This activity corresponds to protease D.

Step 5: chromatography on DEAE-Sephadex. The pooled fractions from the previous step comprising proteases A and B were applied to a DEAE-Sephadex A-50 column (1.3 × 4.5 cm) previously equilibrated in 20 mM Tris · HCl (pH 7.5). The column was washed with 0.3 M KCl/ 20 mM Tris · HCl (pH 7.5). Fractions of 3.4 ml were collected. The proteolytic activities eluted with 50 ml of a linear gradient of 0.3—1.0 M KCl in 20 mM Tris · HCl (pH 7.5), collecting fractions of 1 ml (Fig. 2). With this procedure and in relation to the previous step a purification of 3 and 3.8 times of proteases A and B was accomplished,

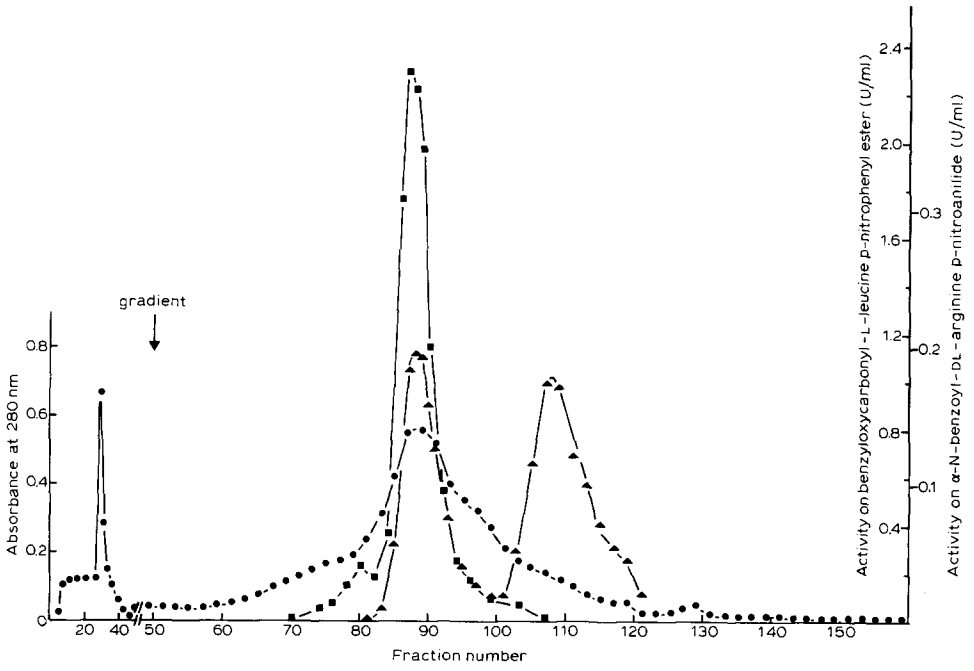

Fig. 1. Chromatography on DEAE-SH-cellulose of three proteolytic activities present in *Artemia* larvae. Step 4 of purification as described in the text. ●——●, absorbance at 280 nm; ■——■, activity on benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester; ▲——▲, activity on α-*N*-benzoyl-DL-arginine *p*-nitroanilide.

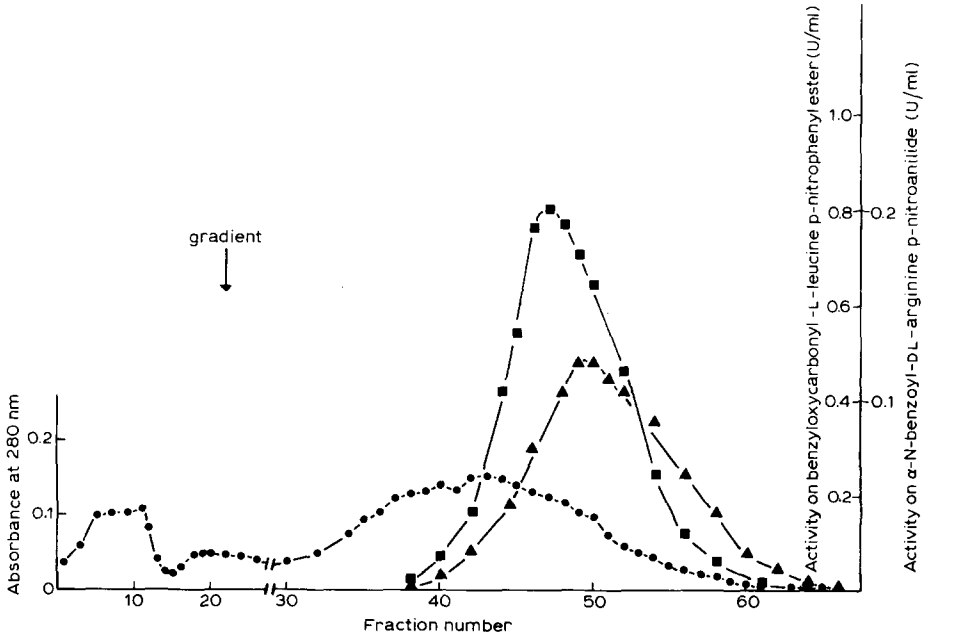

Fig. 2. Chromatography on DEAE-Sephadex of proteases A and B. Step 5 of purification as described in the text. ●——●, absorbance at 280 nm; ■——■, activity on benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester; ▲——▲, activity on α-*N*-benzoyl-DL-arginine *p*-nitroanilide.

TABLE I

Purification of three proteases A, B and C from extracts of *Artemia* larvae of 60 h. The results are set apart for proteases A, B and C. At early steps (1, 2, 3) proteases B and C are not separated and only a global evaluation of both proteases on α-*N*-benzoyl-DL-arginine *p*-nitroanilide is possible. Proteins were determined by the method of Lowry [11].

PROTEASE A

Step	Volume (ml)	Protein (mg)	Activity * (units)	Specific activity (U/mg)	Yield (%)
1. 150 000 × *g* supernatant	30	414	32.1	0.072	100
2. $(NH_4)_2SO_4$ concentration	8.7	141.8	25.4	0.179	79
3. Sephadex G-100 chromatography	158	30.6	22.6	0.745	70
4. DEAE-SH-cellulose chromatography	29	6.9	16.1	2.32	50
5. DEAE-Sephadex chromatography	29.4	1.8	12.2	6.96	38

PROTEASES B AND C

Assuming that the activity on α-*N*-benzoyl-DL-arginine *p*-nitroanilide of the 150 000 × *g* supernatant are due to equal amounts of proteases B and C (see this table), a purification factor of 134 and 305, with recoveries of 56 and 54% are achieved for proteases B and C, respectively.

Step	Volume (ml)	Protein (mg)	Activity (units)	Specific activity (U/mg)	Yield (%)
1. 150 00 × *g* supernatant	30	414	9.06	0.022	100
2. $(NH_4)_2SO_4$ concentration	8.7	141.8	7.46	0.052	82
3. Sephadex G-100 chromatography	158	30.6	6.70	0.223	74
4. DEAE-SH-cellulose chromatography					
Protease B	29	6.9	2.72	0.390	30
Protease C	27.2	0.73	2.47	3.36	27
5. DEAE-Sephadex chromatography					
Protease B	29.4	1.8	2.59	1.47	28

* Determined on benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester.

but not the separation of both activities. Maximum peaks of activity on benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester and α-*N*-benzoyl-DL-arginine *p*-nitroanilide are not coincident. Several attempts were made, all unsuccessful, to separate proteases A and B through chromatography on columns of DEAE-Neocell and hydroxyapatite. A summary of a typical purification run is given in Table I. A purification of 96, 134 and 305 fold, with recoveries of 38, 56 and 54% was accomplished for proteases A, B and C, respectively. Dodecyl sulphate polyacrylamide gel electrophoresis of a pool of proteases A and B (step 5) gave three bands corresponding to proteins with molecular weights of 45 000, 37 000 and 30 000. The two last proteins could correspond to proteases A and B, respectively, as their molecular weights are in good agreement with those

determined by Sephadex G-75 chromatography (see below). The slowest migrating band may correspond to a contaminant protein. Electrophoresis, in the same denaturing conditions, of the fraction enriched in protease C (Step 4) gave two bands corresponding to proteins of molecular weights of 44 000 and 35 000. The former band may correspond to a contaminant protein and the latter to protease C (see below).

Substrate specificity

Protease C is separated from proteases A and B at the DEAE-cellulose step, and its substrate specificity can be easily determined. Protease C hydrolyzes the L-form of α-*N*-benzoyl-DL-arginine *p*-nitroanilide (K_m = 11 μM), (*p*-toluenesulfonyl)-L-arginine methyl ester and azocasein. No activity was detected on benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester. No appreciable inhibition was observed with up to 5 mM phenylmethylsulfonylfluoride, but was strongly inhibited by *N*-α-*p*-tosyl-lysine chloromethyl ketone [4].

Although a net separation of proteases A and B has not been obtained with the different methods used, their substrate specificity could be established using fractions from DEAE-cellulose, DEAE-Sephadex and Sephadex G-75 columns without cross contamination and with the help of phenylmethylsulfonylfluoride, inhibitor at μM concentrations of protease A but not of protease B [4]. Protease A is active on benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester (K_m = 53 μM) but not on α-*N*-benzoyl-DL-arginine *p*-nitroanilide, whereas protease B is active on the L-form of α-*N*-benzoyl-DL-arginine *p*-nitroanilide (K_m = 20 μM), (*p*-toluenesulfonyl)-L-arginine methyl ester, casein and azocasein. The different substrate specificity of proteases A and B can be also appreciated by the different elution profiles of the activities on benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester and α-*N*-benzoyl-DL-arginine *p*-nitroanilide in chromatography on columns of DEAE-SH-cellulose (Fig. 1), DEAE-Sephadex (Fig. 2) and, particularly, Sephadex G-75 (result not shown).

Protease A belongs probably to the chymotrypsin-like serine proteases as hydrolyzes the leucine ester bond of benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester [12,13] and could be classified under EC 3.4.21 corresponding to serine proteases. Although protease B is much less inhibited by phenylmethylsulfonylfluoride than protease A, it is strongly inhibited by *N*-α-*p*-tosyl-lysine chloromethyl ketone [4], a reagent of the histidyl residue present in the active site of certain proteases [14]. As the arrangement Asp-His-Ser seems to be present in the active site of serine proteases, enzyme B could be included also among those belonging to this type. It is also a trypsin-like enzyme as hydrolyzes the amide group of α-*N*-benzoyl-DL-arginine *p*-nitroanilide in which the carboxyl group is apported by arginine [9].

Molecular weight determination

The molecular weight of the different proteases was determined by gel filtration using a Sephadex G-75 column (0.8 × 142 cm) previously equilibrated in 20 mM Tris · HCl, pH 7.5. Cytochrome *c*, myoglobin, chymotrypsinogen and ovalbumin were used as markers. The estimated molecular weights for proteases A, B and C were 38 000, 33 000 and 34 000 respectively. The possibility that the different proteases here described arise from a single protease by autodiges-

tion was investigated through the determination of the molecular weight of the proteases at different stages of purification (see Table I): Steps 2, 4 and 5 for protease A; Steps 4 and 5 for protease B and Step 4 either as specified in Table I or omitting the previous Steps 2 and 3 for protease C. In all cases the values obtained for each protease were essentially equal to the molecular weights described here. It is worthy to recall that the molecular weights of these proteases are slightly higher than most of the known alkaline endopeptidases.

Influence of temperature, sulfhydryl reagents, divalent cations and pH on the activity of proteases A, B and C

Aliquots from pooled fractions of Step 5 (for proteases A and B) in 20 mM Tris · HCl (pH 7.5)/0.7 M KCl and aliquots from Step 4 (for protease C) in the same buffer were heated for 5 min at different temperatures in the presence of 1 mg/ml bovine serum albumin to prevent unspecific surface inactivation. After cooling, the remaining activity was determined using benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester as substrate for protease A, and α-*N*-benzoyl-DL-arginine *p*-nitroanilide for proteases B and C. The temperatures at which half of the activity of proteases A, B and C were inactivated in these experimental conditions were of 60, 52 and 45.5°C, respectively (Fig. 3).

Preincubation of proteases A, B and C with 10 mM mercaptoethanol during 15 min at 4°C, or its presence at 5 mM concentration in the reaction mixture of

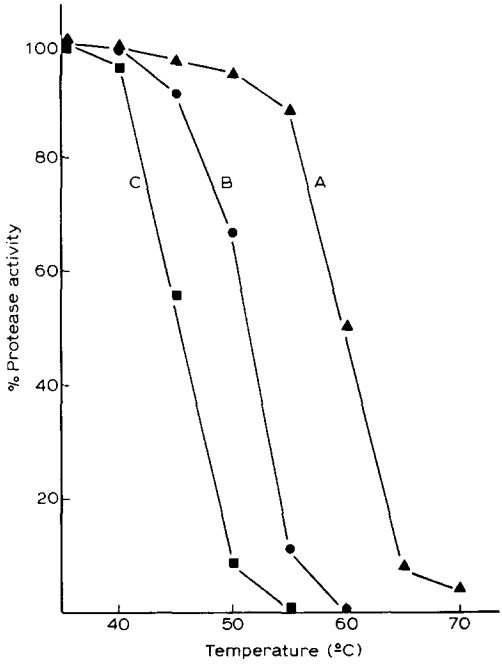

Fig. 3. Influence of temperature on the activity of proteases A, B and C. The experiment was performed as described in the text. ▲———▲, activity on benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester; ●———●, activity on α-*N*-benzoyl-DL-arginine *p*-nitroanilide and ■———■, activity on α-*N*-benzoyl-DL-arginine *p*-nitroanilide.

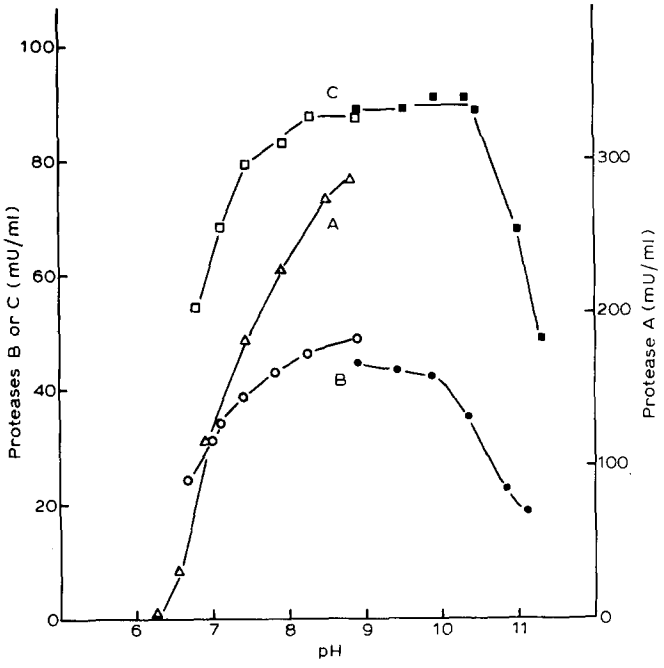

Fig. 4. Influence of pH on the activity of proteases A, B and C. The enzyme preparation used was from Step 5 for proteases A and B and from Step 4 for protease C. pH values were obtained in 40 mM Tris · HCl buffer (△, ○, □) or in 20 mM glycine/NaCl/NaOH (●, ■). The activity given for protease A was calculated considering both the variation of the molar extinction coefficient of *p*-nitrophenol as a function of pH and the spontaneous hydrolysis of benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester at the different pH values used. The enzymatic activity could not be measured beyond pH 9 as the non enzymatic hydrolysis of the substrate was too high in those conditions. The total substrate consumption at the points of maximal rates was less than 20% of that present at zero time.

proteases B and C did not affect their enzymatic activity. Protease A can not be assayed in the presence of 5 mM mercaptoethanol and benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester, as the substrate is rapidly degraded in these conditions. Concentrations of up to 0.1 mM *p*-hydroxymercuribenzoate did not affect significantly the activity of any of the three proteases.

Proteases A, B and C do not require Mg^{2+} or Ca^{2+} ions. Aliquots of Step 4 were extensively dialyzed for 24 h at 4°C against 20 mM Tris · HCl (pH 7.5)/5 mM EDTA, changing the buffer several times. No loss of activity was observed for any of the three proteases when assayed in the absence of divalent cations. A slight inhibition of protease C (10%) occurred in the presence of 10 mM concentrations of Mg^{2+} or Ca^{2+}.

Proteases A, B and C are optimally active at alkaline pH (Fig. 4). Protease A was assayed in the presence of benzyloxycarbonyl-L-leucine *p*-nitrophenyl ester and at pH 6.3—8.8. No activity was detected at pH 6.3 and the velocity increased steadily as the pH of the reaction mixture was more alkaline. The influence of pH on proteases B and C was assayed in the presence of α-*N*-benzoyl-DL-arginine *p*-nitroanilide. In both cases a broad peak of activity was obtained between values 7.5 and 10. These enzymes differ from lysosomal pro-

teases [15] and from other previously described *Artemia* proteases [16] in their alkaline pH optimum.

Studies are in progress to dilucidate the role of these proteases during development. Probably they participate in the degradation of yolk platelets (refs. 2 and 3 and Olalla et al., unpublished data) and in the modification and degradation of *Artemia* RNA polymerases [5,17].

Acknowledgments

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