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Purification and characterization of hatching enzyme from brine shrimp *Artemia salina*

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By using *Artemia* chorion as a specific substrate, the hatching enzyme from *Artemia salina* (AHE) was purified by gel-filtration and ion-exchange chromatography, and characterized biochemically and enzymatically in this study. It was found that the AHE had a molecular weight of 82.2 kDa by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and often contained 73.3 kDa molecules in preparation. The AHE had obvious choriolytic activity, which was optimal at pH 7.0 and a temperature of 40°C. The K_m value of the AHE for dimethyl casein was 8.20 mg/ml. The AHE activity was almost completely inhibited by soybean trypsin inhibitor and *p*-amidinophenyl methane sulfonyl fluoride hydrochloride, greatly inhibited by *N*-tosyl-L-lysyl chloromethyl ketone, phenylmethanesulfonyl fluoride, and lima bean trypsin inhibitor, slightly inhibited by pepstatin, *N*-tosyl-L-phenylalanyl chloromethyl ketone, leupeptin, *N*-ethylmaleimide, and iodoacetamide, and not inhibited by chymostatin and bestatin. All these results imply that AHE is most probably a trypsin-type serine protease. Besides of these, AHE was also sensitive to EDTA and Zn^{2+}. Combined with the results that the EDTA-pre-treated HE activity could be perfectly recovered by Zn^{2+}, it is indicated that AHE might be also a kind of Zn-metalloprotease.

Keywords hatching enzyme; *Artemia salina*; choriolytic activity; trypsin-type serine protease; metalloprotease

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Introduction

Hatching enzyme (HE), secreted from hatching gland cells in hatching embryos for digesting their protective extracellular coats, is used as an important tool in the process of enzymatic hatching in many animal species [1–7]. The HE provides a typical model in the studies of certain cell differentiation, specific protein synthesis, and special gene expression regulation during a certain stage of early embryos at the morphological and molecular level. It will be of great importance to understand its biochemical properties and gene structure in terms of embryogenesis and embryo pharmacology.

The biochemical properties of HE from many animal species, such as mammalian [4], avians [8], amphibians [6,7,9], teleostean [2,3,10,11], echinoderm [1,5], and insect [12], had been studied since 1980s. In invertebrates, HEs of sea urchin had been elucidated in terms of their functional properties, molecular structures, and gene structure [1,13–15]. Recently, HEs of shrimp *Penaeus chinensis* have been successfully purified and characterized biochemically and enzymatically [16]. By now, the cDNAs of HEs from sea urchins *Paracentrotus lividus* and *Hemicentrotus pulcherrimus* [13,14], medaka fish [17], Japanese eel *Anguilla japonica* [18], clawed toad *Xenopus laevis* [19], and Japanese quail *Coturnix japonica* [8] had also been successfully cloned. Researches on animal HEs become more and more comprehensive and profound recently.

The brine shrimp, living in worldwide salt lakes and sea, has many significant roles besides being food for the birds, fishes, and other animals. They assist in the clean up of the lake and ocean by ridding the waters of contaminants such as phosphorous, nitrogen, and other household waste products. Unfortunately, there are no reports on the purification and characterization of the HEs from brine shrimp by now. This study is aimed to purify HE from hatching embryos of brine shrimp *Artemia salina* (AHE) and characterize it biochemically and enzymatically with chorion fragments as the specific substrate.

Materials and Methods

Materials

Dried cysts of *A. salina* were obtained from Enter Biotechnology Co., Ltd (Qingdao, China). Dimethyl casein, proteinase inhibitors, bovine serum albumin (BSA), and other reagents were all purchased from Sigma (St. Louis, USA).

Preparation of chorions

About 10,000 brine shrimp cysts were hydrated in tap water for 30 min at room temperature, followed by treatment with 2.5% NaClO for 30 min to remove the outer carapaces. After being washed with distilled water (DW) to remove leftover NaClO, the cysts were collected and squeezed with a size-4 syringe needle. After being washed twice with DW, the collected chorions were dissociated into fragments ultrasonically. The fragments were collected, dehydrated with acetone for 10 min, washed twice with DW, and then centrifuged at 2000 *g* for 45 min at 4°C. The sedimented fragments were suspended and adjusted to 1000 eggs' chorion/ml with 20 mM Tris–HCl buffer (pH 7.5), and stored at −20°C until use.

Preparation of AHE

About 5×10^5 brine shrimp cysts were hydrated in tap water for 30 min at room temperature, followed by treatment with 2.5% NaClO for 30 min to remove the outer chorions. After washed with DW to remove NaClO, the cysts were collected and cultured in seawater fortified with penicillin (200 IU/ml) and streptomycin sulfate (200 μg/ml) at 31°C. The hatching medium was collected by filtration with a nylon sieve after 90% of embryos hatched out and then precipitated with 67% saturated $(NH_4)_2SO_4$ solution (67P-SAS) at 4°C. After centrifugation (9000 *g*, 30 min, 4°C), the pellet was dissolved and dialyzed with 20 mM Tris–HCl buffer (pH 7.5), and concentrated to 2 ml with Centricon 10 (Amicon, Beverly, USA). The concentrated dialysate was used as crude AHE preparation.

Purification of AHE

After concentrated by Centricon 10 (Amicon), the crude AHE was loaded onto a Sephacryl S-100 gel-filtration column (1.1 cm × 45 cm; Pharmacia, San Francisco, USA) and eluted with Tris–HCl buffer (pH 7.5). The fractions were collected and their hydrolytic activities were measured with 10 mg/ml dimethyl casein. The fractions having high hydrolytic activity were also bioassayed with fragmented egg chorions (1000 eggs' chorion/ml). The gel-filtrated, hydrolytically and choriolytically active fractions were pooled together. After concentrated, they were loaded onto a 1.1 cm × 25 cm DEAE-Sepharose FF column (Pharmacia), eluted with an increased concentration of 0.5 M NaCl in 20 mM Tris–HCl buffer (pH 7.5). The fractions were collected and their hydrolytic and choriolytic activities were measured as described below. The hydrolytically and choriolytically active fractions were pooled, desalted, concentrated, and used as purified HE. All the chromatographic manipulations were performed at 4°C.

Proteolytic activity assay

The proteolytic activity of AHE on dimethyl casein was measured according to Yasumasu *et al*. [2]. Briefly, to 10 μl of the enzymatic solution (in 20 mM Tris–HCl, pH 7.5), 10 μl of 10 mg/ml dimethyl casein (in 20 mM Tris–HCl, pH 7.5) was added and mixed thoroughly. After incubated at 35°C for 30 min, the reaction was stopped by adding 0.3 ml of 100 mg/ml ice-cold trichloroacetic acid (TCA). After centrifugation, the supernatant was measured with an UV-2450 Spectrophotometer (Shimadzu, Kyoto, Japan) at 280 nm. The proteolytic activity estimated as the increment rate of absorbance of 0.001/min was taken to be 1 U:

$$\text{Activity} = A_{280} \times 10^{-3}/\text{min}.$$

Choriolytic activity assay

The choriolytic activity of AHE was determined by using fragmented egg chorion as a specific substrate according to Li *et al*. [16], and modified as follows: 100 μl of chorion fragments (1000 eggs' chorion/ml) was mixed with 100 μl AHE solution. After incubated at 35°C for 30 min, the reaction was stopped by adding 2.8 ml of 100 mg/ml ice-cold TCA. After centrifugation, the supernatant was measured with an UV-2450 Spectrophotometer (Shimadzu) at 280 nm. The choriolytic activity was estimated the same as mentioned above.

Determination of the optimal temperature and pH of AHE

The temperature profile of AHE in 20 mM Tris–HCl (pH 7.5) was determined in a temperature range of 20–60°C. The pH profile of AHE was determined in a pH range between pH 5.0 and 10.0. NaAc–HAc buffer was used for pH 5.0, Na_2HPO_4–NaOH buffer for pH 6.0 and 7.0, Tris–HCl buffer for pH 8.0, and Gly–NaOH buffer for pH 9.0 and 10.0. The choriolytic activity was measured spectrophotometrically under the same conditions as described above.

Kinetic studies

Kinetic parameters were determined by the Lineweaver–Burk plot method as described by Lepage and Gache [1]. One hundred microliters of dimethyl casein from 0.5 to 10 mg/ml were added to 100 μl of purified AHE, respectively, and the enzymatic activities of AHE were measured spectrophotometrically under the same conditions as described above.

Effect of inhibitors and ions

To 10 μl of purified AHE sample, 10 μl of each inhibitors and ions, such as soybean trypsin inhibitor (SBTI), *p*-amidinophenyl methane sulfonyl fluoride hydrochloride (*p*-APMSF), *N*-α-tosyl-L-lysine chloromethyl ketone

(TLCK), phenylmethanesulfonyl fluoride (PMSF), lima bean trypsin inhibitor (LBTI), pepstatin A, leupeptin, tosyl-L-phenylalanine chloromethyl ketone (TPCK), *N*-ethylmaleimide (NEM), iodoacetamide (IAM), chymostatin, bestatin, ethylene diamine tetraacetic acid (EDTA), and Ca^{2+}, Mg^{2+}, Zn^{2+}, and Cu^{2+} (Sigma-Aldrich, St Louis, USA) was added, respectively, and incubated at 30°C for 30 min. Then 10 μl of 10 mg/ml dimethyl casein was added to each sample and incubated at 30°C for 30 min. The proteolytic activity was measured spectrophotometrically under the same conditions as described above. In the reaction mixture, AHE substituted by Tris–HCl buffer (pH 7.5) was used as a negative control, whereas inhibitors and ions substituted by Tris–HCl buffer (pH 7.5) was used as a positive control, and the 280 nm absorbance of positive control was designated as 100%.

To 10 μl of purified AHE pre-incubated with 20 mM of EDTA for 30 min at 4°C, 10 μl of 10 mM different metal ions was added, respectively, and incubated for 30 min at room temperature. After 10 μl of 10 mg/ml dimethyl casein was added and mixed thoroughly, the mixture was incubated for 30 min at 35°C and measured spectrophotometrically under the same conditions as described above. The enzyme activity of AHE pre-incubated with no inhibitors or metal ions was used as the positive control.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to Laemmli [20]. And 10% gels and low molecular protein markers (Dongfeng Biotechnology Co., Ltd, Shanghai, China) from 14.4 to 97.4 kDa were used. Proteins separated by SDS–PAGE were silver stained according to Wray et al. [21].

Protein concentration assay

Protein concentrations of the different AHE preparations were measured according to Bradford [22]. BSA was used as a protein standard.

Statistical analysis

Data are expressed as mean±SD of triplicates and analyzed for statistical significance by using one-way analysis of variance. Values of $P<0.01$ were considered significant.

Results

Purification of AHE

Two milliliters of crude AHE, with total protein content of 17.12 mg and total choriolytic activity of 192.00 U, were collected and concentrated for column chromatography.

To a Sephacryl S-100 gel-filtration column, 0.2 ml of concentrated crude AHE was loaded [**Fig. 1(A)**]. Fractions with high choriolytic activities (fractions 22–24) were collected and pooled together to 5.7 ml finally. The pooled fractions had a total protein content of 2.19 mg and total choriolytic activity of 69.00 U. SDS–PAGE showed that the peak fractions contained a large amount of 73.3 kDa molecules and a small amount of 82.2 kDa molecules after silver staining (Fig. 2).

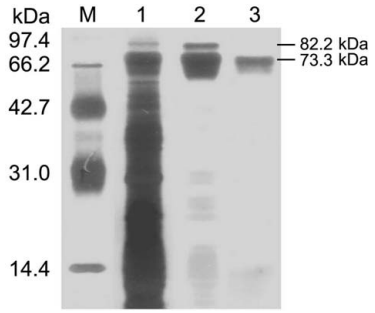

Figure 2 Silver staining of different HE preparations from *A. salina*
Lane M, SDS–PAGE middle molecular protein standards with molecular weight (kDa) indicated in the left; lane 1, crude AHE; lane 2, Sephacryl S-100 fractions 22–24; and lane 3, DEAE-Sepharose FF fractions 15–16. About 12.5% gel was used.

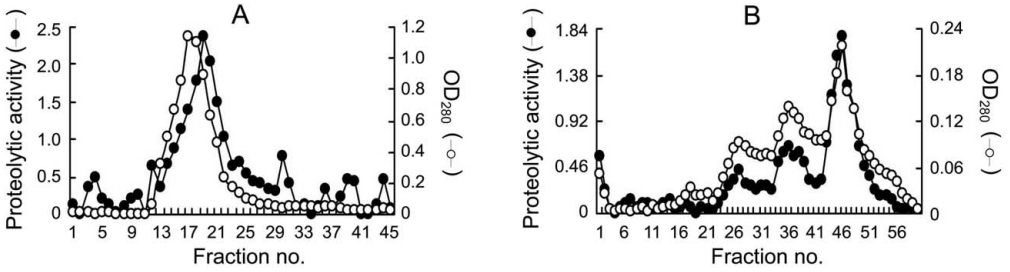

Figure 1 Column chromatography of crude HE from *A. salina* (A) The Sephacryl S-100 column chromatography of crude AHE. Fractions 22–24 contained the highest choriolytic activity. (B) DEAE-Sepharose Fast Flow column chromatography of the Sephacryl S-100 run-off fractions. Fractions 15–16 contained the highest choriolytic activity. The proteolytic activity was measured by using 10 mg/ml dimethyl casein as a substrate. The choriolytic activity was measured by using fragmented chorion (1000 eggs' chorion/ml) as a specific substrate. Profiles corresponding to the enzyme activity and absorbance at 280 nm are shown.

Table 1 Purification of AHE

Step	Total protein (mg)	Total activity[a] (U)	Specific activity[a] (U/mg)	Recovery (%)	Purification
Crude AHE[b]	17.12	192.00	11.21	100	1.00
Sephacryl S-100 fraction	2.19	69.00	31.66	36	2.82
DEAE-Sepharose FF fraction	0.12	48.00	400.00	25	35.67

[a]The choriolytic activity of AHE was measured by using fragmented chorion (1000 eggs' chorion/ml) as a specific substrate; and [b]derived from 5×10^5 *Artemia* embryos. AHE, *Artemia* hatching enzyme.

To a DEAE-Sepharose anion-exchange column, 5 ml of pooled fractions of gel filtration were applied [**Fig. 1(B)**]. The eluted fractions (fractions 15–16) in the peak with high choriolytic activities were collected and pooled together to 1.8 ml finally. The pooled fraction had a protein content of 0.12 mg and total choriolytic activity of 48.00 U. The SDS–PAGE assay showed that the peak fractions contained only 73.3 kDa molecules after silver staining (**Fig. 2**).

After purification, the specific choriolytic activities of AHE molecules derived from Sephacryl S-100 gel-filtration and DEAE-Sepharose anion-exchange were 2.82 and 35.67 folds higher than that of crude AHE obtained from 67P-SAS precipitates, respectively (**Table 1**). Recovery was 25% of the crude AHE.

Optimal temperature and pH

The effects of different temperatures and pH values on the choriolytic activity of purified AHE are shown in **Fig. 3**. The optimal temperature of the purified AHE was about 40°C [**Fig. 3(A)**], and its optimal pH value was about 7.0 [**Fig. 3(B)**].

Enzyme kinetics

The purified AHE was incubated together with dimethyl casein at different concentrations from 0 to 10 mg/ml, and its proteolytic activity was measured spectrophotometrically (**Fig. 4**). The K_m value of AHE for dimethyl casein was calculated as 8.20 mg/ml.

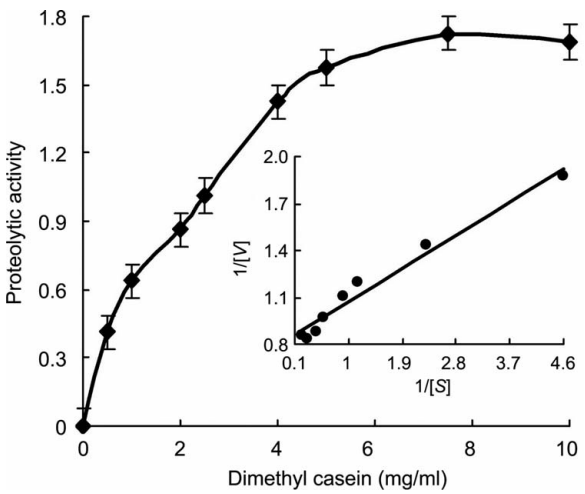

Figure 4 Kinetic properties of purified HE from *A. salina* The K_m values were determined by using dimethyl casein as a substrate. According to the Lineweaver–Burk model, the K_m was calculated to be 8.20 mg/ml.

Inhibition studies

The effects of different protease inhibitors, EDTA, and some metal ions were examined with respect to the proteolytic activity of the purified AHE on dimethyl casein. **Table 2** shows that the AHE activity was almost completely inhibited by SBTI and *p*-APMSF, greatly inhibited by TLCK, PMSF, and LBTI, and slightly inhibited by pepstatin A, leupeptin, TPCK, NEM, and IAM, but not inhibited by bestatin and chymostatin. Besides of these, the AHE

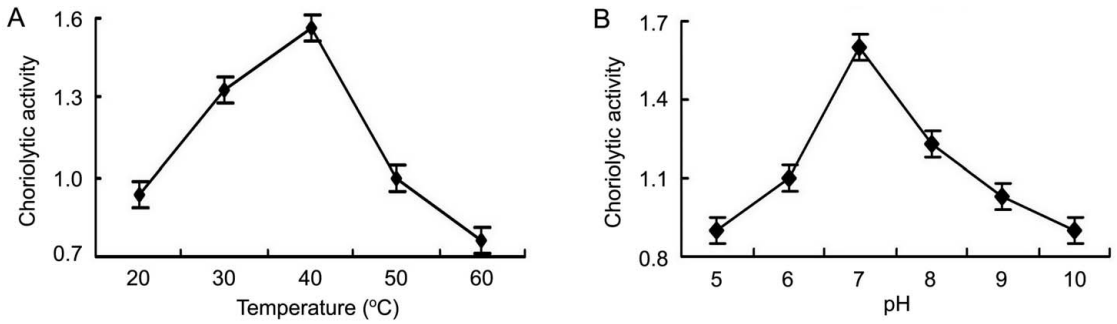

Figure 3 Effects of different pH and temperatures on purified HE from A. salina (A) Effect of different temperatures on AHE activity. The optimal temperature of the enzyme was 40°C. (B) Effect of different pH values on AHE. The optimal pH value of the enzyme was about 7.0. The choriolytic activity was measured by using fragmented chorion (1000 eggs' chorion/ml) as a specific substrate.

Table 2 Effects of inhibitors, chelators, and metal ions on purified AHE

Ion or inhibitor	Concentration (mM)	Maximum inhibition (%)
SBTI	5.00	96.15 ± 0.24
p-APMSF	10.00	92.31 ± 0.59
TLCK	50.00	73.08 ± 0.15
PMSF	50.00	71.15 ± 0.12
LBTI	5.00	65.39 ± 0.30
Pepstatin A	2.50	18.75 ± 0.34
Leupeptin	2.00	11.54 ± 0.20
TPCK	1.00	11.54 ± 0.19
NEM	25.00	7.69 ± 0.36
IAM	25.00	3.85 ± 0.16
Chymostatin[a]	0.50	0.00 ± 0.01
Bestatin	1.00	0.00 ± 0.01
EDTA	20.00	56.52 ± 0.63
Cu^{2+}	5.00	−24.00 ± 0.29
Mg^{2+}	2.00	2.00 ± 0.24
Zn^{2+}	2.50	−128.00 ± 0.12
Ca^{2+}	0.50	−4.00 ± 0.22

Concentrations of inhibitors and metal ions indicate those exhibiting highest inhibition so far tested. The proteolytic activities were measured by using 10 mg/ml dimethyl casein as a substrate. Values are the mean ± SD of three pooled samples in triplicate. [a]Dimethyl sulfoxide (20%) used as a solvent did not affect the activity.

Table 3 Recovery effect of metal ions on the EDTA-pre-treated AHE

Treatment	Proteolytic activity (%)
HE only	100.00 ± 0.01
HE + EDTA (20 mM)	43.48 ± 0.63
HE + EDTA (20 mM) + Cu^{2+} (10 mM)	32.61 ± 0.85
HE + EDTA (20 mM) + Zn^{2+} (10 mM)	102.17 ± 0.44
HE + EDTA (20 mM) + Ca^{2+} (10 mM)	34.78 ± 0.54
HE + EDTA (20 mM) + Mg^{2+} (10 mM)	26.09 ± 0.62

Values are the mean ± SD of three pooled samples in triplicate.

was inhibited by EDTA, activated by Zn^{2+} and Cu^{2+}, but not influenced by Ca^{2+} and Mg^{2+}.

The recovery effect of metal ions on the activity of EDTA-pre-treated HE was also investigated in this study. The recovery effects of different metal ions are shown in **Table 3**. These results indicated that enzymatic activity of the purified AHE, inhibited by 20 mM EDTA, could be restored almost to the original level by 10 mM Zn^{2+}, whereas other metal ions could not.

Discussion

HEs are pivotal proteases that help the encysted embryos to escape from their egg chorions during hatching process [1,2,4,7]. Crude hatching media contained hydrolytic activities on a variety of synthetic substrates indicating a very complex picture [2,3,6–9]. Because of this complexity, activities with more specific biological relevance were sought. And it was found that the HE from shrimp [16] had not only proteolytic activity but also choriolytic activity, similar with those of echinoderms [1,5], fish [2,3,18], and amphibians [6,7,9,23]. In 1970, Yamagami [24] developed an effective method for HE activity assay based on the increase of 280 nm absorbance of chorionase digested turbid suspension of chorion fragments, and characterized the chorionase activity of HEs in *Oryzias latipes* successfully. In 2006, Li *et al*. [16] used a modified method based on Yamagami and characterized the choriolytic activity of *Penaeus* HE successfully. As egg chorion is the natural substrate of AHE, fine fragments of chorion from the embryos of *A. salina* were employed to evaluate the specific activity of AHE in this study.

Purification of HE has been reported in many vertebrates and also invertebrates, however, the purification of crustacean HE has been rarely reported because the HE was usually very difficult to collect and of very small amount [1,2,9,12,16]. In this study, AHE was first purified successfully from *Artemia* hatching medium by column chromatography and characterized biochemically and enzymatically by using fragmented egg chorions as specific substrates. The AHE purification scheme was successful, in that the purified AHE gave a single band in SDS–PAGE after silver stained and the specific activity increased about 35.67 times compared with the crude AHE, with an overall recovery of 25% of the initial activity.

SDS–PAGE assay showed that the estimated molecular mass of the purified AHE was 73.3 kDa which had both high proteolytic and choriolytic activity. It was also found that the molecular weight of AHE was larger than that of HEs from sea urchins (37–51 kDa) [1,5,25], shrimp *P. chinensis* (43 kDa) [16], sea-squirt *Ciona intestinalis* (34 kDa) [26], fish (15–40 kDa) [2,3,11,18,27–29], and amphibians (50–65 kDa) [6,7,9,23].

The optimal pH of AHE for choriolytic activity was at around 7.0, which was lower than that of HE from sea urchin (pH 8.0) [1], sea-squirt (pH 8.5) [26], and fish HEs (pH 8.0–8.7) [2,3,27,28], higher than that of shrimp (pH 6.0) [16], but similar to that of amphibians (pH 7.4–7.6) [6,7]. The temperature optimum of AHE was about 40°C, which was identical to that of shrimp HE [16], higher than that of fish HEs (30–35°C) [2,3,11,27,28], amphibian HEs (30°C) [6,7], and sea urchin (35°C) [1]. The K_m value of AHE for dimethyl casein was calculated as 8.20 mg/ml, which was higher than that of the HE from shrimp *P. chinensis* (7.47 mg/ml) [16] and flounder *Paralichthys olivaceus* (4.28 mg/ml) [11]. The diversities of the optimum temperatures, pH, and K_m

values may be correlated with the difference in species, their survival environments, and enzyme structures, the ion concentrations as well.

The proteolytic activity of AHE was almost completely inhibited by SBTI and *p*-APMSF, and greatly inhibited by TLCK, PMSF, and LBTI. This indicates that AHE might be a kind of trypsin-type serine protease, which was similar to HEs from shrimp *P. chinensis* [16], flounder *P. olivaceus* [11], killifish *Fundulus heteroclitus* [28], Zebrafish *Brachydanio rerio* [30], toad *X. laevis* [7], and mouse [4], but different from some fish HEs, a kind of trypsin-type alkaline protease [2,3,27], frog *Rana pirica* HE, a kind of trypsin-type cysteine protease [6], and sea urchin *Strongylocentrotus purpuratus* HE, a kind of chymotrypsin-like protease [31].

The AHE was sensitive to EDTA and several metal ions. Combined with the results that its EDTA-pre-inhibited activity could be perfectly recovered by Zn^{2+}, it is indicated that AHE might be also a kind of Zn^{2+}-metalloprotease, which is similar to that of HEs from marine sponge *H. pulcherrimus* [32], sea urchin *S. purpuratus* [31], sea-squirt *C. intestinali*s [26], shrimp *P. chinensis* [16], flounder *P. olivaceus* [11], medaka fish *O. latipes* [2,3], killifish *F. heteroclitus* [28], pike *Esox lucius* [29], toad *X. laevis* [7], and frog *R. pirica* [6]. The AHE has almost the same property of metalloprotease as HE from *P. chinensis* [16] but the latter was more sensitive to EDTA and strongly inhibited by Zn^{2+}, Ca^{2+}, Mg^{2+}, and Cu^{2+}.

In summary, the AHE, for the first time, was purified and characterized as a trypsin-type serine protease and a Zn^{2+}-metalloprotease as well, with the molecular weight of about 73.3 kDa on SDS−PAGE, and its choriolytic activity was optimal at pH 7.0 and a temperature of 40°C, respectively. Efforts are currently being made to clone the AHE gene and to characterize its temporal and spatial expression patterns in the process of embryo hatching.

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