PURIFICATION AND CHARACTERISATION OF A VITELLOGENIN DERIVED AMINOPEPTIDASE FROM RAINBOW TROUT EGGS

Jakub KRONOVETR[a,b1,*], Jana BARTHOVÁ[a1], Tomislav BARTH[b2] and Helena RYŠLAVÁ[a2]

[a] *Department of Biochemistry, Faculty of Science, Charles University, 128 40 Prague 2, Czech Republic; e-mail:* [1] *barthova@prfdec.natur.cuni.cz,* [2] *rysl@prfdec.natur.cuni.cz*

[b] *Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, 166 10 Prague 6, Czech Republic; e-mail:* [1] *kubak@uochb.cas.cz,* [2] *barth@uochb.cas.cz*

Received March 14, 2000
Accepted April 26, 2000

An aminopeptidase specific for N-terminal alanine was isolated from the rainbow trout (*Oncorhynchus mykiss*) maturing eggs using a simple procedure. The enzyme is composed of two identical units each of MW 16 000, separable by disulfide reduction. The enzyme has a high content of acid amino acids and its N-terminal sequence is the following ^{1}EVNAVKCSMV^{10}RDTLTTFNNK^{20}KYQIN^{25}. The sequence is identical with that of the rainbow trout vitellogenin precursor beginning with the amino acid in position 1 385. No peptidase activity has been so far observed in proteins derived from this precursor. The enzyme activity is partially blocked by Na_4-EDTA but it is not inactivated by 4-(chloromercuri)benzoate, phenylmethanesulfonyl fluoride or pepstatin A.

Key words: Enzymes; Aminopeptidases; Fish eggs; 4-Nitroanilides; Rainbow trout; Vitellogenin.

Maturing fish eggs release various types of enzymes into the surrounding water. These enzymes are responsible for the final rupture of the egg envelope, enabling the mature embryo to leave the egg[1–6] and also for the protection of embryos against bacterial and fungal infection[7–10]. There is good evidence that one family of these enzymes are endopeptidases[11–13]. Our earlier experiments[14–16] proved that the water in which the eggs were incubated contained enzymes that were able to hydrolyse various chromogenic substrates (aminoacyl-4-nitroanilides) for aminopeptidases. In this contribution we describe the isolation and partial characterisation one of the enzymes that hydrolyse alanine-4-nitroanilide.

EXPERIMENTAL

Materials

Eggs: Rainbow trout eggs were obtained from the trout hatchery in Liběchov (Czech Republic) ten days before hatching; they were frozen and stored at –20 °C.

Chemicals: Amino acid and peptide 4-nitroanilides (pNA), substrates of different exo- and endopeptidases were the following: Cys(Bzl)-pNA, Bz-Arg-pNA, Pro-pNA, Leu-pNA, Arg-pNA, N-glutaryl-Phe-pNA, Z-Gly-Pro-pNA, Ala-pNA, Phe-pNA, Tyr-pNA. These compounds were synthesised at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic. Superdex S-75 10/30, Concanavalin A-Sepharose 4B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Molecular weight standards for electrophoresis, pI standards, gels and staining kits were obtained from Pharmacia Biotech AB (Uppsala, Sweden). Phenylmethanesulfonyl fluoride (PMSF), molecular weight standards for Superdex column calibration, pepstatin A and Immobilon P^{SQ} membrane, were purchased from Sigma (St. Louis, U.S.A.), Na_4-EDTA, *N*,*N*-dimethylformamide (DMFA) and natrium salt of 4-(chloromercuri)benzoate (PCMB) from Lachema (Brno, Czech Republic).

Methods

Enzyme isolation: The content of trout eggs was released by scission of the egg envelope in highly purified (mQ) water (0.5 ml per egg) and soluble proteins were separated from the precipitate by centrifugation (7 000 *g*, 15 min, 4 °C). Before collecting the supernatant, the yolk sack oil globules were mechanically removed from the supernatant surface. The supernatant was freeze-dried and stored at –20 °C. The lyophilised sample (2 mg) was dissolved in 200 μl of 20 mM phosphate buffer (pH 8) and applied onto Superdex S-75 10/30 column equilibrated with the same buffer on Waters–FPLC system (flow rate 0.5 ml/min, eluted proteins were detected by measuring absorption at 276 nm). The alanine aminopeptidase activity was measured in the fractions. Fractions hydrolysing alanine 4-nitroanilide were collected and rechromatographed on the same column under the same conditions.

Peptidase activity determination. The reaction mixture contained 20 mM phosphate sodium buffer (pH 8), 0.33 mM amino acid 4-nitroanilide, 0.3% DMFA and additional agents such as inhibitors, if tested. The reaction was started by adding approximately 45 nmol of enzyme. The concentration of 4-nitroaniline released from amino acid 4-nitroanilide substrates in 2–18 h at 25 °C was measured at 405 nm. The specific activity (U/mg) was calculated as $a = V\,\Delta A/\varepsilon\, d\, t\, m$ (V, volume of reaction mixture (l); ΔA, the change in absorbance at 405 nm; ε, absorption coefficient for 4-nitroaniline ($1.02 \cdot 10^{-2}$ l/μmol cm); d, cuvette diameter (cm); t, time of reaction (min); m, weight of protein (mg)).

Electrophoresis. SDS PAGE and blotting of proteins was performed on PhastSystem apparatus (Pharmacia Biotech AB 1995) following User manual. Isoelectric focusing was performed in the range of pH 3–9 on the same device. Reduction of disulfide bonds of proteins was carried out with 2-mercaptolethan-1-ol (5%) at 100 °C for 5 min. Gels were silver-stained[17,18].

N-terminal sequence analysis. N-terminal amino acid sequence analysis was performed after electrophoretic separation from a piece Immobilon P^{SQ} membrane with blotted enzyme on LF 3600 Protein Sequencer (Beckman) using Edman degradation[19,20].

Amino acid analysis. Proteins were hydrolysed in 6 M HCl for 24 h and analysed on Amino Acid Analyser Biochrom 20 (Pharmacia). Cysteine was estimated after oxidation with performic acid (0 °C, 4 h) as cysteic acid.

Affinity chromatography. Affinity chromatography on Concanavalin A Sepharose was performed according to Bittiges *et al.*[21].

MALDI-TOF mass analysis. Molecular weight was determined on a Biflex analyser.

Determination of protein concentration was performed according to Bradford[22].

RESULTS AND DISCUSSION

Several recent papers[14–16] presented the occurrence of aminopeptidase activity in the medium, where fish eggs were cultured. We also observed a high aminopeptidase activity inside the egg and the eggs were the source of the enzyme we have studied. The specific activity of enzyme from the culture medium was rather higher than that from eggs, but the amount of the enzyme there was negligible. The enzyme isolation from the eggs seems to be better way because of the simple method we developed.

The precipitation of ballast proteins of the egg content resulted in fifty-fold purification of the enzyme hydrolysing Ala-pNA. Trout egg proteins soluble in mQ water were chromatographed on a Superdex S-75 10/30 column (see Fig. 1). The elution profile of proteins was monitored by mea-

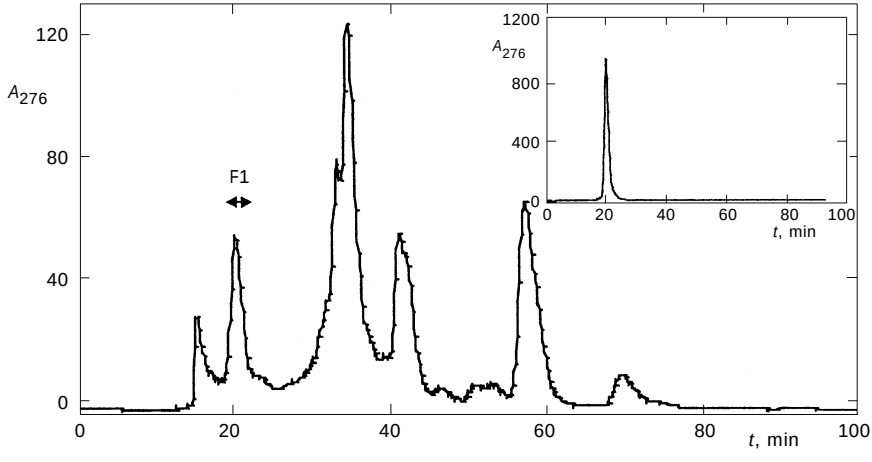

FIG. 1

Chromatography of trout egg proteins soluble in mQ water on the Superdex S-75 10/30 column. A lyophilized sample (2 mg) was dissolved in 200 µl of 20 mM phosphate buffer (pH 8) and applied onto a Superdex S-75 10/30 column equilibrated with the same buffer in the Waters–FPLC system (flow rate 0.5 ml/min, eluted proteins were detected at 276 nm). F1, alanine aminopeptidase activity. Inset: rechromatography on a Superdex S-75 10/30 column of protein fraction hydrolysing Ala-pNA

suring absorbance at 276 nm and the alanine aminopeptidase activity. The enzyme activity was found in the peak with elution time around 20 min. The fractions showing enzyme activity were collected, lyophilised and rechromatographed under the same conditions (see Fig. 1, inset). The specific activity of the isolated enzyme was 130 times higher than that found in the eggs and was calculated to be $1.82 \cdot 10^{-2}$ U/mg. The purification procedure of the enzyme was checked by SDS electrophoresis (see Fig. 2).

The molecular weight of the enzyme in non-reduced form was estimated by chromatography on a Superdex column calibrated by molecular weight standards, by SDS PAGE and by MALDI-TOF methods, giving values 33 000, 33 000 and 36 297–36 751, respectively. The discrepancy between the values obtained by chromatography and mass spectrometry might have been caused by some interactions between the saccharide component of a glycoprotein with dextran in a Superdex column or with SDS in electrophoresis. The electrophoretic results, show that the form with reduced S–S bonds has MW 16 000. According to our results, the alanine aminopeptidase is a homodimeric molecule where monomers are S–S covalently bound.

The isoelectric point of the pure enzyme lies between 5.8 and 7.7. We observed ten protein bands in this range. We assume that individual enzyme

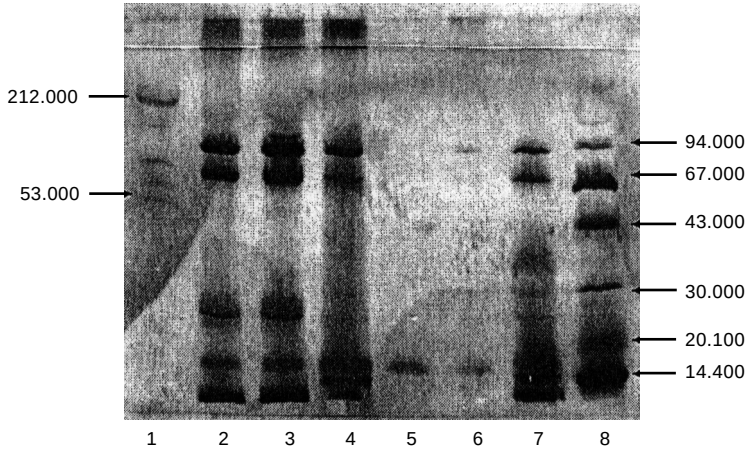

FIG. 2

SDS PAGE of trout eggs proteins. 1, 8 molecular weight standards; 2, 3 trout egg extract; 4 trout egg proteins soluble in mQ water; 5 alanine aminopeptidase after rechromatography; 6, 7 proteins released from rainbow trout eggs into the surrounding water during incubation at different development stages (6 one day after ovulation, 7 one day before hatching). Gel "grad 10-15" was silver stained

molecules differ in their charged moiety, in the amount of sialic acid[23,24] or sulfonic or phosphoric acid groups[25] in the molecule.

Enzyme has no affinity to Concanavalin A-Sepharose, which is able to bind glycoproteins with mannoside structures[21]. Therefore we assumed the presence of sialic acids on O-glycosidically bound sugar. Sialic acids obviously terminate saccharide chains in some glycoproteins[25]. The fact that the enzyme does not bind to Concanavalin A-Sepharose does not exclude the presence of N-glycosidically bound saccharides.

The enzyme specificity was measured using ten different 4-nitroanilide substrates; a very high specificity for Ala-pNA was observed. Generally, aminopeptidase specificity is usually quite low, alanine, valine and leucine substrates are hydrolysed at the same rate by one aminopeptidase. We observed a difference in the rate of hydrolysis when arginine (10%) and leucine (2%) substrates were taken into consideration. The reaction rates were related to the rate of hydrolysis of Ala-pNA (100%). The rates of hydrolysis of other used substrates (amino acid or peptide 4-nitroanilide, see Materials) were very low (less than 0.5%).

The enzyme activity (substrate Ala-pNA) was not blocked by pepstatin A, PMSF and only slightly by PCMB. 1 mM EDTA reduced enzyme activity to 30% (see Table I). The alanine aminopeptidase from rainbow trout eggs could be a member of the metalloprotease family[26].

When we compared the N-terminal amino acid sequence ^{1}EVNAVKCSMV^{10} RDTLTTFNNK^{20}KYQIN^{25} with data from SwissProt database[27] we found it to be almost identical (except glutamine instead of lysine in position 23) to the rainbow trout vitellogenin precursor[28]. The vitello-

TABLE I
Inhibition of alanine aminopeptidase activity

Inhibitor	Concentration	Activity, %
None	–	100
Pepstatin A	1 μM	100
Phenylmethanesulfonyl fluoride	1 mM	110
4-(Chloromercuri)benzoate	1 mM	84
Na_4-EDTA	1 mM	35

genin amino acid sequence was read from cDNA. Vitellogenin is the precursor of major egg-yolk proteins witch are sources of nutrients during early development of oviparous organisms. It is produced by liver, secreted into blood and then transported by endocytosis into growing oocytes, where it is generally cleaved into smaller products[29]. This enzyme could be one of them. Vitellogenin is an estrogen-induced egg protein, used for determination of water contamination[30,31].

Amino acid analysis of Ala-aminopeptidase showed that acid amino acids (and their ω-amides) made 24% of the content, neutral lipophilic Ile, Leu and Val gave the same value. The arginine content in contrast to lysine is low, the same is true for histidine (2.6%) (see Table II).

From the hatching enzyme studies of other authors[2,4–6,32] it follows that the enzyme function occurs only several hours before hatching in the egg. We detected the enzyme activity a couple of days after fertilisation. Rainbow trout eggs have one month incubation period. The enzyme we studied was probably not a hatching enzyme, although we observed its choriolytic activity (data not shown). The enzyme could also change the envelope structure during the embryo development (water hardening)[33,34]. We cannot exclude that the alanine aminopeptidase participates in the hatching.

TABLE II
Amino acid analysis of alanine aminopeptidase from rainbow trout eggs

Amino acid	mole%	Amino acid	mole%	Amino acid	mole%
Asx	14.3	Cys	4.6	Phe	3.2
Thr	4.5	Val	7.1	His	2.6
Ser	6.5	Met	3.3	Lys	9.1
Glx	9.1	Ile	5.2	Trp	0.6
Gly	6.5	Leu	7.8	Arg	2.6
Ala	4.6	Tyr	3.9	Pro	4.5
				Total	100

REFERENCES

1. Schoots A. F. M., Denucé J. M.: *Int. J. Biochem.* **1981**, *13*, 591.
2. Ishida J.: *Zool. Sci.* **1985**, *2*, 1.
3. Yasumasu S., Iuchi I., Yamagami K.: *Zool. Sci.* **1988**, *5*, 191.
4. Yasumasu S., Iuchi I., Yamagami K.: *J. Biochem. (Tokyo)* **1989**, *105*, 212.

5. Yasumasu S., Iuchi I., Yamagami K.: *J. Biochem. (Tokyo)* **1989**, *105*, 204.
6. Post L. L., Schuel R., Schuel H.: *Biochem. Cell. Biol.* **1988**, *66*, 1200.
7. Kudo S., Inoue M.: *Zool. Sci.* **1986**, *3*, 323.
8. Kudo S., Inoue M.: *J. Exp. Zool.* **1991**, *259*, 392.
9. Kudo S., Inoue M.: *Naturwissenschaften* **1991**, *78*, 172.
10. Kudo S.: *Experientia* **1992**, *48*, 277.
11. Yamagami K.: *Zool. Sci.* **1996**, *13*, 331.
12. Lee K. S., Yasumasu S., Nomura K., Iuchi I.: *FEBS Lett.* **1994**, *339*, 281.
13. Carnevali O., Carletta R., Cambi A., Vita A., Bromag N.: *Biol. Reprod.* **1999**, *60*, 140.
14. Anzenbacherová E., Barth T., Barthová J., Hamáčková J., Kouřil J. in: *Measures for Success* (J. Muir and F. Sevilla, Eds), p. 137. Bordeaux Aquaculture, Bordeaux 1994.
15. Kronovetr J., Barthová J., Barth T., Anzenbacherová E., Hamáčková J., Kouřil J.: *Pol. Arch. Hydrobiol.* **1998**, *45*, 331.
16. Barth T., Barthová J., Vacek J., Kronovetr J., Hamáčková J., Kouřil J., Hauzerová L. in: *Towards Predictable Quality* (L. Laird and H. Reinersten, Eds), p. 8. European Aquaculture Society, Trondheim 1999.
17. Laemmli U. K.: *Nature* **1970**, *227*, 680.
18. Heukeshoven J., Dernick R.: *Electrophoresis* **1985**, **6**, 103.
19. Edman P., Begg G.: *Eur. J. Biochem.* **1967**, *1*, 80.
20. Edman P.: *Mol. Biol. Biochem. Biophys.* **1970**, *8*, 211.
21. Bittiges E. H., Schnebli H. P.: *Concanavalin A as a Tool*. J. Wiley and Sons, London 1976.
22. Bradford M. M.: *Anal. Biochem.* **1976**, *72*, 248.
23. Kitazume S., Kitajima K., Inoue S., Inoue Y.: *Anal. Biochem.* **1992**, *202*, 25.
24. Kitajima K., Nomot H., Inoue Y., Iwasaki M., Inoue S.: *Biochemistry* **1999**, *23*, 310.
25. Stryer L.: *Biochemistry*, 4th ed., Chap. 18. W. H. Freeman and Co., New York 1995.
26. Barrett A. J. in: *Proteinase Inhibitors* (A. J. Barrett and G. S. Salvesen, Eds), Chap. 1. Elsevier Science Publishers BV, Oxford 1986.
27. Bairoch A., Apweiler R.: *Nucleic Acids Res.* **1999**, *27*, 49.
28. Mouchel N., Trichet V., Bletz A., Le Pennec J. P., Wolff J.: *Gene* **1996**, *174*, 59.
29. Lafluer G. J., Jr., Byrne B. M., Kanungo J., Nelson L. D., Greenberg R. M., Wallace R. A.: *J. Mol. Evol.* **1995**, *41*, 505.
30. Heppell S. A., Denslow N. D., Folmar L. C., Sullivan C. V.: *Environ. Health Perspect.* **1995**, *103*, 9.
31. Knudsen F. R., Schou A. E., Wiborg M. L., Mona E., Toleffsen K., Stenersen J., Sumpter J. P.: *Bull. Environ. Contam. Toxicol.* **1997**, *59*, 802.
32. Yamagami K. in: *Fish Physiology* (W. S. Hoar and D. J. Randall, Eds), Vol. XI, Chap. 7. Academic Press, San Diego 1988.
33. Ha C. R., Iuchi I.: *J. Biochem (Tokyo)* **1997**, *122*, 947.
34. Ha C. R., Iuchi I.: *J. Biochem (Tokyo)* **1998**, *124*, 917.