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The Mode of Action on the Vitelline Envelope of *Xenopus* Hatching Enzyme as Studied by Its Two Molecular Forms

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ABSTRACT—The medium in which dejellied prehatching *Xenopus laevis* embryos were cultured (hatching medium) can solubilize the vitelline envelope (VE) of dejellied eggs and dimethyl casein. Western blot analysis using antibodies against the hatching enzyme revealed the presence of 60 kDa and occasionally 40 kDa molecules in the hatching medium. Ion-exchange chromatography or gel-filtration followed by enzyme activity assays indicated that the fractions containing 60 kDa molecules exhibit VE-solubilizing activity but those containing 40 kDa alone do not, although both fractions exhibit the same level of proteolytic activity. However, solubilization of the VE was obtained when the 40 kDa fraction was mixed with an extremely low concentration of the 60 kDa fraction that cannot solubilize VE by itself, or when the 40 kDa fraction was applied to the VE that had been pretreated with a low concentration of the 60 kDa fraction. We propose that recognition and/or processing of the VE by 60 kDa molecules make solubilization of the VE possible by 40 kDa molecules.

INTRODUCTION

The hatching of *Xenopus* embryos is aided by an embryo-derived enzyme, i.e., a hatching enzyme, which partially digests the fertilization envelope and jelly (Carroll and Hedrick, 1974; Yoshizaki and Yamasaki, 1991). Efforts have been made to isolate the hatching enzyme (Urch and Hedrick, 1981), but its detailed characterization has not been fully successful primarily due to an extremely small amount of enzyme produced by the embryos. Recently, we have determined the primary structure of the *Xenopus* hatching enzyme based on the cloned cDNA (Katagiri *et al.*, 1997), and thereby obtained the specific antiserum that can inhibit the VE-solubilizing activity of the hatching medium. In a further effort to isolate the hatching enzyme activity using these antibodies as a probe, we found two molecular forms that seem to behave in different ways in solubilizing the VE. A possible mechanism of the solubilization of VE by the hatching enzyme was indicated by the results of experiments using these different forms of molecules.

MATERIAL AND METHODS

Preparation of hatching enzyme

Sexually mature African clawed frogs, *Xenopus laevis*, were induced to ovulate by injection of human chorionic gonadotropin. Fertilized eggs were obtained by artificial insemination, cultured in 1/10 Steinberg's solution, and were staged according to Nieuwkoop and Faber (1967). Embryos at stage 19-20 were dejellied with 2.5% thioglycolate (pH 8.3) in an agar-based dish. Dejellied embryos were thoroughly rinsed, and their fertilization envelopes were removed by forceps. Denuded embryos were cultured in Steinberg's solution for 24 hr at 24°C until they attained stage 35/36. The medium in which embryos had been cultured ("hatching medium") was collected, concentrated by Centricut 10, dialyzed against deionized water, and lyophilized.

The lyophilates were dissolved in 50mM Hepes-NaOH (pH 7.4) for precipitation with 67% saturation of $(NH_4)_2SO_4$ (SAS). The precipitate (67% SAS ppt.) was dissolved in 50 mM Hepes-NaOH (pH 7.4), and was subjected to gel-filtration on a Superdex 75 (Pharmacia), or ion-exchange chromatography on a Mono Q (Pharmacia) column.

Assay of enzyme activity

Mature unfertilized eggs were dejellied with thioglycolate. Six dejellied eggs were placed in 0.3 ml test solutions, and were observed at 10 min-intervals to determine the time it took for their vitelline envelope to be completely solubilized. To determine the proteolytic activity, each 20 μl test solution (in 50 mM Hepes-NaOH, pH 7.4) and 0.1% dimethyl casein were incubated at room temperature (20-25°C) for 30 min. Incubation was terminated by the addition of 1.05 ml of cold H_2O and 0.45 ml of 0.01% fluorescamine for fluorometric determination with excitation at 390 nm and emission at 475 nm.

Electrophoresis and Western blotting

SDS-PAGE was carried out according to Laemmli (1970). Western blotting was carried out using the antiserum (anti-GST-UVS.2) specific to *Xenopus* hatching enzyme, as described elsewhere (Katagiri *et al.*, 1997). Proteins separated on SDS-PAGE were transblotted to a polyvinylidiene difluoride membrane (ImmobiconTM). After blocking with 5% dry-milk, the membrane was incubated with 1:1,000 dilution of anti-GST-UVS.2 serum and 1:500 dilution of alkaline phosphatase-conjugated goat anti-rabbit IgG, followed by development with a BCIP/NBT phosphatase system.

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RESULTS AND DISCUSSION

When dejellied eggs were placed in the crude hatching medium or its 67% SAS precipitates at a concentration of 500-600 embryos/ml, vitelline envelopes (VEs) became swollen within 15 min, and were dissolved completely after 40-60 min. When dejellied fertilized or activated eggs were treated with the hatching medium under the same conditions, fertilization envelopes were wrinkled and ruptured followed by removal away from the eggs within 60 min, but were not solubilized completely. Because of the ease with which the enzymatic action was witnessed, VEs from unfertilized eggs were used as the substrate for the hatching enzyme in the following experiments. On Western blotting, the enzymatically active preparations exhibited either 60 kDa alone or both 60 kDa and 40 kDa bands according to different preparations (Fig. 1). Regardless of the relative amounts of 60 kDa and 40 kDa components in each preparation, there was no distinct difference with respect to the VE-solubilizing activity.

The hatching medium from 1,500 embryo cultures was gel-filtrated on a Superdex 75 column (Fig. 2). Peaks of fractions exhibiting proteolytic activity against dimethyl casein were pooled as described in the legend for Fig. 2, Western blotted, and were tested for VE-solubilizing activity. Groups III and IV, but not others, displayed a strong VE-solubilizing activity and positive 60 kDa and 40 kDa components in Western blots (Fig. 3A).

In anion-exchange chromatography on a Mono Q column, the highest proteolytic activity was eluted at 0.1-0.2 M NaCl fraction (Fig. 4). On Western blotting, these fractions exhibited only 40 kDa components, and 60 kDa components were not observed in any fractions (Fig. 3B). Assays on dejellied eggs revealed, however, that no fractions, including those exhibiting highest proteolytic activity, possessed VE-solubilizing activity.

The results presented above suggest the necessity of 60 kDa molecules for successful digestion of VE. To confirm this, the 67% SAS precipitates were fractionated by either a Superdex 75 or a Mono Q column, and the concentration of the fractionated samples was equalized to 1,000 embryos/ml. Determination of proteolytic activity against dimethyl casein revealed approximately the same level of activity in both Superdex 75 and Mono Q fractions. Western blotting showed the occurrence of both 60 kDa and 40 kDa components in

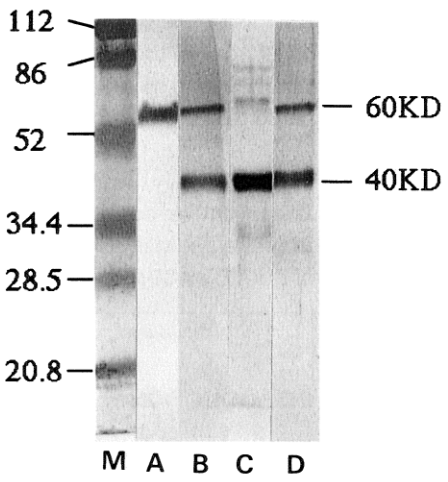


Fig. 1. Western blotting of hatching enzyme preparations using anti-GST-UVS.2 antiserum as a probe. A, B, crude hatching medium; C, D, 67% SAS precipitates; M, molecule markers with molecular weights (KD) to the left.

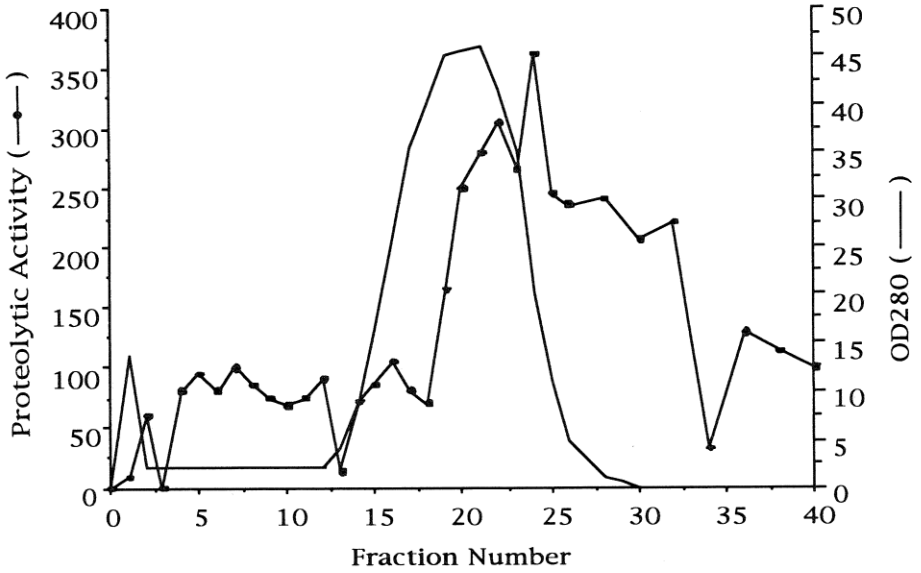


Fig. 2. Gel-filtration of the hatching medium from a 1,500 embryo culture on a Superdex 75 column. The column was equilibrated with 50 mM Hepes-NaOH (pH 7.4), and eluted at a flow rate of 0.5 ml/tube/min. Proteolytic activity was assayed using 0.1% dimethyl casein as a substrate. Fractions were pooled in groups I-VII as follows: group I (fr. 11-13), group II (fr. 14-18) , group III (fr. 19-22), group IV (fr. 23-25), group V (fr. 28-30), group VI (fr. 31-34), and group VII (fr. 35 , 36). Western blot of group III is shown in Fig. 3A.

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Superdex 75 fractions, and only 40 kDa components in Mono Q fractions (Fig. 3). Assays using dejellied eggs showed that the Superdex 75 fraction had a strong VE-solubilizing activity, but the Mono Q fraction did not (Table 1). It was noticed, however, that in Mono Q fractions, the VE became swollen after 100-120 min, suggesting certain effects exerted by this fraction.

Since the Mono Q fraction possessed proteolytic activity although it did not digest the VE, 40 kDa molecules contained in this fraction were expected to cooperate with the Superdex 75 fraction in the solubilization of the VE. To address this question, the Mono Q fraction was mixed with the Superdex 75 fractions at an extremely low concentration that could not solubilize the VE by itself. The results presented in Table 2 indicate that the Superdex 75 fraction, which exhibited no VE-solubilizing activity, could solubilize the VE when mixed with the Mono Q fraction. Thus, 40 kDa molecules contained in the Mono Q fraction participate in solubilization of the VE.

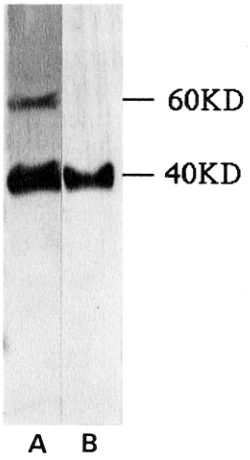


Fig. 3. Western blotting of the hatching medium after fractionation by Superdex 75 (A) and Mono Q (B), as shown in Fig. 2 and Fig. 4.

In an attempt to examine the relative roles of 60 kDa and 40 kDa molecules in the solubilization of the VE, we treated dejellied eggs with the Superdex 75 fraction (24 embryos/ml) for 10 min, washed them with De Boer's solution, and transferred them to the Mono Q fraction (1,000 embryos/ml). Treatment with the Superdex 75 fraction for 10 min did not affect VE visually at all, but the post-treatment with the Mono Q fraction resulted in VE solubilization (Table 3). These results indicate that 40 kDa molecules require a precedent processing of the VE mediated by 60 kDa molecules in order to successfully solubilize the VE.

The cooperative action of 60 kDa and 40 kDa molecules described above poses the question of whether these molecules represent different enzymes acting synergitically for the solubilization of the substrate envelope, as found in the hatching enzyme of fish (Yasumasu *et al.*, 1988). The preparation containing 60 kDa alone can readily solubilize the VE, suggesting that proteolytic activity exhibited by 40 kDa molecules also exists in 60 kDa molecules. In support of this notion is the observation that the Superdex 75 and Mono Q fractions share hydrolytic activities against quite similar synthetic substrates as well as sensitivity to protease inhibitors

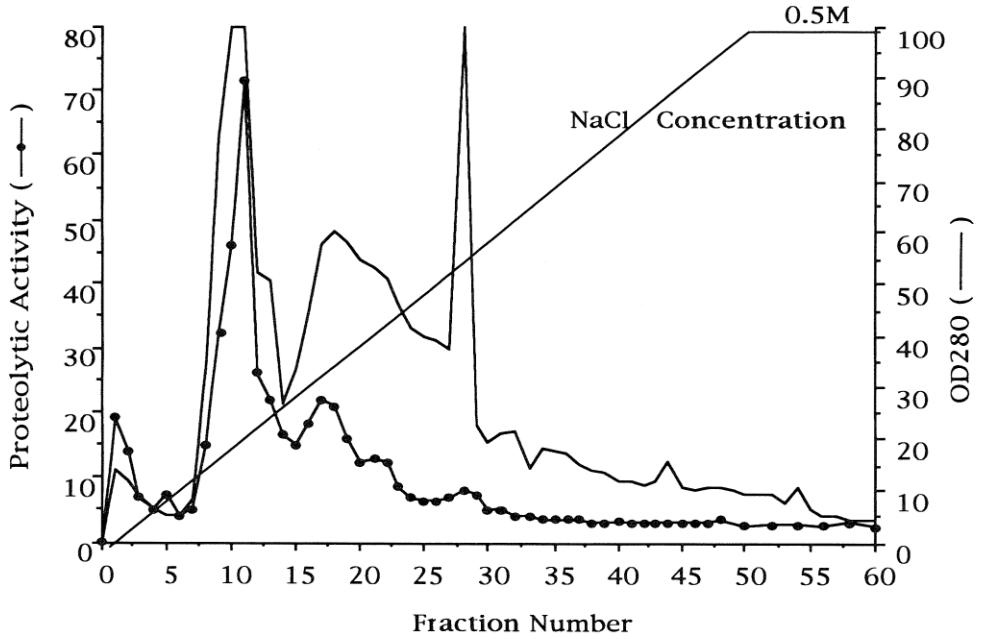


Fig. 4. Fractionation of the hatching medium on a Mono Q HR 5/5 column. The 67% SAS precipitate derived from 3,000 embryo culture was applied on the column equilibrated with 50 mM Hepes-NaOH (pH 7.4), and eluted with a linear gradient of NaCl at a flow rate of 1.0 ml/tube/min. Proteolytic activity was assayed using 0.1% dimethyl casein as a substrate. Fractions 8-11 were pooled for Western blotting, as shown in Fig. 3B.

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Table 1. Comparison of VE-solubilizing and proteolytic activities between Mono Q and Superdex 75 fractions

Fraction	Number of eggs with VE digested at			Proteolytic activity[1)]	Immunoreactive molecule[2)]
	30 min	40 min	120 min		
Mono Q	0	0	0	98.3	40 kDa
Superdex 75	3	6		102	60 kDa, 40 kDa

[1)] Assayed on dimethyl casein.
[2)] Based on Western blotting.

(unpublished data). Thus, we propose that the Mono Q fraction represents the protease domain which is retained after degradation or autodigestion of larger (60 kDa) molecules during manipulation. This assumption is plausible in view of the primary structure of the *Xenopus* hatching enzyme which was recently revealed to have two repeats of CUB domain, each comprising 110 amino acids toward the C-terminal side of the metalloprotease domain(Katagiri *et al.*, 1997). Assuming that CUB repeats function in recognition and/or processing of the conformation of the VE or the enzyme itself, then the 40 kDa Mono Q fraction possessing proteolytic activity represents the metalloprotease domain localized at the N-terminal side of 60 kDa molecules. Substantiation of this assumption requires further purification of both 60 kDa and 40 kDa molecules, and studies along this line are currently under way.

Table 2. Effect of the addition of Superdex 75 fraction on VE-solubilizing activity of Mono Q fraction from the hatching medium

Treatment [1)]	Number of eggs with digested VE at		
	40 min	50 min	120 min
Mono Q fraction (1000)	0	0	0 [2)]
Superdex 75 fraction (24)	0	0	0 [2)]
Mono Q (976) & Superdex 75 (24)	2	6	

[1)] Numbers in parentheses indicate concentrations expressed as the number of embryos/ml.
[2)] VEs were swollen.

Table 3. Effect of pretreatment of the VE with Superdex 75 fraction on VE-solubilizing activity of Mono Q fraction

Treatment [1)]	Number of eggs with VE digested at		
	80 min	90 min	120 min
Q (1,000)	0	0	0 [2)]
S (24)	0	0	0 [2)]
S (20), 10 min → Q (1,000)	2	6	
S (20), 10 min → De Boer	0	0	0

[1)] Numbers in parentheses indicate concentrations expressed as the number of embryos/ml.
[2)] VEs were swollen.

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