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The Hatching Enzyme From Xenopus laevis: Limited Proteolysis of the Fertilization Envelope

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Hatching in the amphibian Xenopus laevis involves release of an embryo-secreted hatching enzyme, a protease, which weakens the envelope surrounding the embryo. The envelope is not totally solubilized, which infers that only selected envelope components are hydrolyzed by the enzyme. The susceptibility of the glycoprotein components composing the envelope to hydrolysis by the hatching enzyme was investigated. Isolated envelopes in various physical states, ie, particulate and solubilized, were treated with the hatching enzyme, and the resulting envelope hydrolysis products were characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The susceptibility of the envelope components to proteolysis was not a function of the state of the envelope. The envelope components most susceptible to proteolysis were the 125K and 118K components followed by the 60K and 71–77K components. These components are minor constituents of the envelope. The major constituents, 33K and 40K, were relatively resistant to hydrolysis by the hatching enzyme. From these observations, we infer that the envelope components hydrolyzed are components that link or bind together the major structural elements of the envelope, eg, the 33K and 40K components. Selective destruction of the components required for maintaining the structural integrity of the envelope, eg, the "nuts and bolts" of the structure, permits a weakening of the envelope that allows the embryo to hatch without having to destroy totally (hydrolyze) the envelope.

Key words: hatching enzyme, proteases, limited proteolysis, Xenopus, envelopes

The hatching process in the African clawed toad, Xenopus laevis, has been described as a two step process [1, 2]. The first step involves the escape of the embryo from outer jelly coats, J_3 and J_2; the second step consists of hatching from the innermost jelly layer, J_1, and from the fertilization envelope (FE). The first step appears to involve a physical mechanism wherein J_1 and the perivitelline space swell due to water imbibition and rupture the outer two layers. The J_1 layer then appears to dissolve slowly or erode away, leaving

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the embryo primarily encapsulated by the FE. At the time of hatching from the FE, the embryo uses muscular movement to exit the FE through a weakened spot or bleb on the FE [2]. The formation of a bleb was postulated by Bles [1] to result from enzymatic activity, and Carroll and Hedrick found proteolytic activity released from the embryo at the time of FE hatching [3]. The bleb that forms on the FE is located in close apposition to the head of the embryo, and this particular region of the embryo has been shown to contain hatching gland cells, the presumptive site of hatching enzyme(s) origin [4]. This hatching process is construed as a limited proteolysis of structurally important components of the FE, thus allowing the embryo to rupture the resulting weakened structure by muscular movement and escape.

A proteolytic activity against FE, found coincident with the developmental time of hatching and identified as hatching enzyme (HE), has been purified and characterized as an active-site serine protease with a neutral pH optimum and limited proteolytic activity toward its natural substrate, the FE [5]. In X laevis, the FE is well characterized in terms of its macromolecular components [6]. In other organisms, eg, medaka, mouse, and sea urchin, the analogous envelope structures are less well understood in terms of their chemical nature. The FE has been shown to be composed of some 11 glycoprotein macromolecules derived from the vitelline envelope (VE), a cortical granule lectin (CGL), and a jelly component from J_1 [6]. The change at fertilization causing the block to further sperm entry appears to involve the following: 1) a hydrolytic event changing VE to VE* with a concomitant change in the molecular weight of two macromolecular components [7]; 2) the release of the lectin from the granules, which diffuses through the VE, binds a jelly component in J_1, and forms a macromolecular and cellular block called the F layer [8, 9, 10]. The VE* has markedly different physical properties than the VE; it "melts" at a higher temperature than the VE and is relatively insoluble in solutions of mercaptoethanol in contrast to the VE [11]. It is possible to solubilize the CGL and J_1 (F layer) from the FE by a galactose-EDTA treatment thereby separating the F layer from the insoluble VE* [12]. In view of the above, we approached the question of what components of the envelopes are susceptible to HE hydrolysis using isolated particulate envelopes and solubilized envelope components.

MATERIALS AND METHODS

HE from X laevis was prepared according to the method previously published [5]. The HE used for this work was the supernatant fraction from 10,000–20,000 dejellied embryos that was first lyophilized and then dialyzed at pH 4.0 and low ionic strength resulting in a 9-fold purification. Envelopes were collected according to Wolf et al [6]. Envelope and envelope fractions were iodinated using bovine lactoperoxidase following the procedures of Phillips and Morrison [13]. Bovine lactoperoxidase was purified according to the method of Rombauts et al [14]. Carrier-free Na-^{125}I was obtained from Amersham-Searle. The various fractions iodinated were: 1) intact isolated FE; 2) vitelline envelope (VE) and FE solubilized at 72°C for 5 min in 0.05 M Tris at pH 9.5 and designated "heat dissolved" VE and FE. This dissolution process produces soluble supramolecular complexes of unknown structure [6] However, it does not dissociate the envelope into its individual components, a process which requires a dissociating agent such as SDS; 3) 0.5 M galactose and 0.5 mM EDTA-treated FE, a process known to remove the F layer from the outside of the FE [12]; and 4) heat treatment of FE at 72°C for 1 min in 0.05 M Tris, pH 9.5, resulting in partial solubilization of the FE.

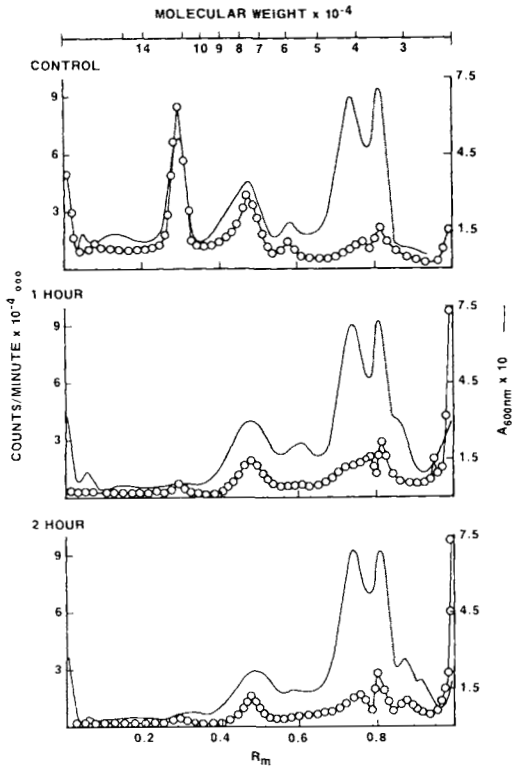

Fig. 1. Hydrolysis of heat-dissolved VE by the hatching enzyme. At 0, 1, and 2 h, VE hydrolysis was assayed by SDS gel electrophoresis. The stained gels were scanned at A_{600nm} and then were sliced for radioactivity measurements (see text). A molecular weight axis at the top of figure is included, derived from electrophoresis of standard proteins.

Proteolysis reaction mixtures contained 70 μg of envelopes mixed with HE and 0.01 M imidazole, pH 8.0, to a final volume of 150 μl and kept for 1 or 2 h at room temperature. The reaction was stopped by adding the Laemmli sample buffer and heating [15]. Electrophoretic separation utilized 8.5% polyacrylamide gels (30:1 ratio of monomer to methylene bis acrylamide) and was performed according to Laemmli's procedures [15]. The gels were fixed and stained by the methods of Fairbanks et al [16]. R_ms were measured from gels scanned at A_{600nm} with the Gilford model 2400-S spectrophotometer [6]. For radioactivity determination, the gels were sliced into 1.0 mm sections with a Mickel gel slicer, and radioactivity of the sections was measured in a scintillation counter.

RESULTS

The SDS-gel patterns of heat-solubilized VE as a function of HE hydrolysis time are given in Figure 1. The figure clearly shows hydrolysis of several different peaks and regions. The peak at 120K (actually a doublet at 125K and 118K) was completely hydrolyzed in 1 h. The material with molecular weights of 71–77K and 60K was hydrolyzed but at a slower rate. The loss of radioactivity in the 71–77K and 60K regions was greater than the decrease in stained material, suggesting only partial hydrolysis of these glycoproteins. The

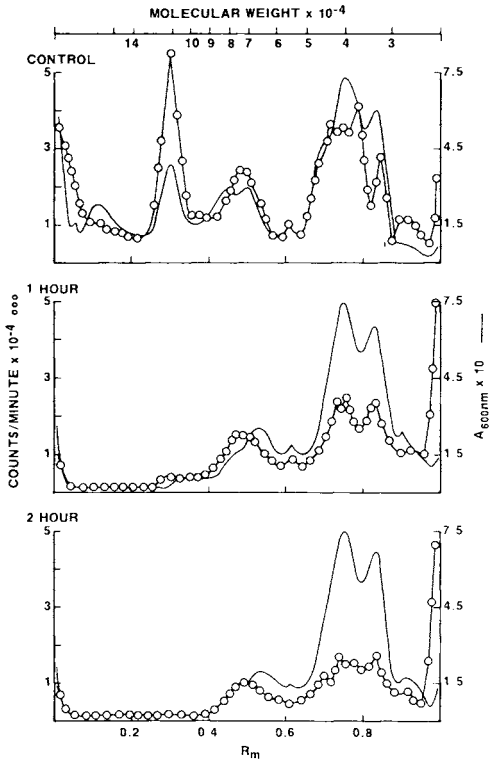

Fig. 2. Hydrolysis of heat-dissolved FE by the hatching enzyme. Conditions as in Figure 1.

top of the gel contains envelope material that was not completely dissociated by SDS, 2-mercaptoethanol, and heating, that was also hydrolyzed by HE. With the hydrolysis of the 120K doublet peak and other components in the profile, there was a concomitant rise in radioactivity at the dye front. Finally, the large doublet at 40K and 33K, the major components of VE, was unaffected.

HE hydrolysis of heat-dissolved VE* was investigated (profile not shown). Complete hydrolysis of the 120K doublet peak was obtained, along with partial hydrolysis of the protein components at 71–77K and 60K as in VE hydrolysis. The only difference from the VE experiment (Fig. 1) was the hydrolysis of the 33K peak components to the extent of about 40%. It is interesting that HE also cleaves material at 71–77K and that there is 71–77K material left after transformation from VE to VE*. The marked differences in the physical properties of these envelopes are not seen in the hydrolysis patterns by HE, except for the 33K peak, which may reflect a conformational change in that molecule. Other than for this difference, the patterns of hydrolysis of solubilized VE and VE* are very similar.

When heat-dissolved FE was hydrolyzed by HE, the same hydrolysis patterns were obtained as above with VE and VE* in that the 120K doublet was completely hydrolyzed, as well as partial hydrolysis of material at 71–77K and 60K (Fig. 2). In addition, hydrolysis of material at 88K and 41–46K was observed.Since these R_ms corresponded to the CGL components, purified CGL was radioiodinated and subjected to hydrolysis by HE; 40% of ^{125}I-CGL was hydrolyzed in 1 h.

TABLE I. Macromolecular Composition of VE and FE†

Mol wt $\times 10^{-3}$	VE %	FE %
125	10.9	11.2
118		
(88)		(0.5)
77	3.8	–
71	4.1	4.8
67	–	5.4
60	2.8	1.2
(41–46)	–	(16.5)
40	38.6	39.1
33	33.4	32.2
30	1.0	0.9
29	2.0	1.8
26	1.7	1.3
< 16	1.7	2.2
Total	100.0	100.1 (117.1)

†Those components in brackets are derived from the CGL contribution of the F layer to the FE. Taken from [6].

Since the above information was collected using heat-dissolved envelopes, and since the intact FE is the natural substrate, the intact FE was iodinated and subjected to hydrolysis by HE. With the intact FE, both the hydrolysis products released and those remaining insoluble were examine by SDS gel electrophoresis. The radioactive electrophoretic patterns obtained from intact FE were quite similar to heat solubilized FE. After 2 h of hydrolysis, approximately 10% of the doublet at 120K (and a small percentage of material at 33K) was hydrolyzed by HE. The SDS gel electrophoresis pattern of the hydrolysis supernatant solution showed molecular weight species at 88K and 41–46K, which suggested that the components of the CGL were first liberated from the envelope and then subsequently hydrolyzed. It appears that with intact FE, longer hydrolysis times or more HE would be needed to increase the extent of hydrolysis.

DISCUSSION

Interpretation of the results reported here on the hydrolysis of envelopes by HE is limited by the possibility that labeling envelopes with ^{125}I, in various states, eg, solubilized versus particulate, VE versus FE, produces a state-dependent labeling pattern. Such a situation would compromise the HE hydrolysis patterns of these envelopes. However, observations have been made (T. Nishihara, G. Gerton, and J.L. Hedrick, unpublished observations) which show the state-dependent labeling pattern not to be a problem for the study reported here. The macromolecules composing the VE indicated in Table I appear to be randomly distributed in that most glycoprotein components of the envelope are radiolabeled using the procedures employed here, although not to the same extent (specific activity). The exception is the 40K envelope component which is not labeled in particulate or heat dissolved envelopes. Radiolabeling of the solubilized envelopes is quantitatively different than labeling the particulate envelopes, ie, higher specific activities were obtained.

In the case of the FE, the F layer blocks the access of the peroxidase to the outer surface of the VE component, but the inner envelope surface facing the plasmalemma is labeled in a fashion similar to that found when labeling the FE from which the F layer had been removed, ie, VE*. Therefore, the results reported here for HE hydrolysis patterns of envelopes in different states is not due to variation introduced into the envelopes by the ^{125}I labeling.

A generally uniform hydrolysis pattern of the envelopes was obtained regardless of the state of the envelope, ie, dissolved or particulate. The envelope components most sensitive to HE hydrolysis were those with molecular weights of 125K and 118K (the unresolved 120K range in the figures). The primary sensitivity of these components was observed no matter what form or state of the envelope was used, (dissolved VE, VE*, FE, or particulate FE). As might be expected, when these macromolecular components were solubilized into supramolecular complexes by heating, they were hydrolyzed faster than when they were integrated into the particulate structure of the envelope (solubilized FE compared with particulate FE). The second most sensitive components to HE hydrolysis were those in the molecular weight regions of 60K and 71–77K. The 71–77K components in the VE are known to be altered at fertilization, perhaps by limited proteolysis, and play a key role in altering the properties of the envelope at fertilization [6, 14]. The 33K component appeared to be resistant to HE hydrolysis in the VE, but when the envelope was converted to VE* at fertilization, the 33K component was susceptible to HE. The VE to VE* transition at fertilization is known to produce marked physical changes in the envelope [11]. Perhaps the sensitivity of the 33K component in the VE* is a reflection of a change in the environment around the 33K component. The sensitivity of the CGL to HE hydrolysis was interesting to us considering the somewhat limited specificity of the HE [5] but is probably not of major physiological importance as altering the interaction of VE* components at the time of hatching likely constitutes the most important structural change that effects the "strength" or integrity of the envelope.

As shown in Table I, the major components of the VE and FE are the 33K and 40K glycoproteins, together constituting 71%–72% of the total weight of the envelopes. These major structural units are either not susceptible to hydrolysis (as in the VE) or are partially hydrolyzed (as in VE* and FE) by the HE. The 125K, 118K, 71–77K, and 60K components are the most susceptible to HE hydrolysis and appear to be elements that bind together the major structural components, in a similar fashion that nuts, bolts, or cross members would hold together girders or beams in a bridge. By removal of a few of these joining components, the overall structure may be weakened without frank collapse. Thus HE may be seen as weakening the envelope structure without total destruction (hydrolysis) such that the muscular movement of the embryo can push apart the remaining structural elements. Additional information on the molecular architecture of the envelopes in structural and functional terms is needed in support of this hypothesis.

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