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A Method for Assaying the Jelly-Digesting Activity of the Hatching Enzyme from Frog Embryos

By

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(With 3 Text-figures and 2 Tables)

It has been shown for several amphibian species that the embryos secrete a substance(s), called "hatching enzyme", as an aid to their natural hatching process. The activity of the enzyme can be visualized by the dissolution or removal of the egg-envelopes surrounding the fresh eggs, the vitelline membrane and the jelly layers (Cooper, 1934). However, no further characterization of the enzyme concerned has since been made, mainly because of the lack of an appropriate method for assessing the enzymatic activity. One way for characterizing the enzyme is the use of a variety of synthetic substrates, as recently done by Carroll and Hedrick (1972) for the *Xenopus* enzyme. At the same time an appropriate use of the natural substrate will be of great value in studying the enzymatic system with which both the enzyme and the substrate are not well characterized.

In a series of studies intended to understand the mechanism of production and secretion of a frog hatching enzyme during the course of developmental process, a simple and sensitive method using a natural substrate jelly was developed for detecting enzymatic activity. The present paper will describe and discuss the validity of this method, together with some properties of the enzyme as elucidated by the assay system.

Material and Methods

Grass frogs, *Rana chensinensis*, used in the present study were collected in the vicinity of Sapporo. The hatching enzyme was obtained in the following way: Thousands of the Shumway stage 18 embryos were denuded by 0.2% pronase-E (Kaken Chem. Co.) dissolved in Steinberg's solution. The denuded embryos were allowed to develop in a sterilized Steinberg's solution for 48 hrs at 18°C, until they attained St. 22, the post-hatching stage. The culture medium was collected by decantation of embryos and centrifuged to remove coarse debris. The supernatant was dialyzed against deionized water followed by lyophilization. The lyophilates, highly active in digesting the envelopes of the eggs at early cleavage stages, could be stored at −20°C at least for 1 year without

loss of activity. In most experiments to be described below, a fraction precipitated by 67% saturation of ammonium sulfate (67P) was used, but in some cases 0.1–0.2% crude enzyme solution was employed as an enzyme source.

The inhibitory effects on the enzyme activity were tested with iodo-acetamide (IAA), *p*-chloromercuribenzoic acid (PCMB), and soybean trypsin inhibitor (SBTI). The mixture of enzyme and inhibitor was stood for 10–15 min, and then assayed for the enzymatic activity. The reference proteins employed in the gel-filtration through a Sephadex G-100 column were bovine serum albumin (BSA, mol. wt. 67,000) and ovalbumin (mol. wt. 40,000). Other particular procedures will be given in each experimental part.

Results

Use of "jelly film" as a substrate for assay

The substrate for the enzymatic assay was prepared in the following manner: Two to 3 g of an oviducal tissue from a hibernating gravid female was homogenized with a steel blender in 100 ml of deionized water (pH 7.3). The homogenate was centrifuged to remove tissue debris. A viscous supernatant, mostly composed of oviducal jelly, was poured on the bottom of a Falcon plastic petri-dish (5 cm in diameter) and was quickly thrown out, so as to leave a thin layer of jelly which sticks to the bottom of the dish. The dish was dried overnight at room temperature, and was briefly washed with 2% acetic acid followed by a wash with deionized water. After desiccation, the "jelly film" on the bottom of the petri-dish served as a substrate for the enzymatic assay. As necessary, the thickness of the jelly film might be increased by repeating the whole procedure described above.

Onto the jelly film thus prepared, a drop of the solution to be tested (usually 0.03–0.05 ml) was placed, and was incubated at room temperature or at 37°C in a moist chamber. Applying a drop of the buffer solution without enzyme should be necessary as a control. At the end of the incubation period, the dish was washed briefly with deionized water to remove test solutions, and then stained with 1% amido black or periodic acid-Schiff (PAS) method.

Fig. 1 is a demonstration of the amido black-stained jelly film on which drops of a hatching enzyme solution were given every 5 min and incubated at 22°C. As shown in the figure, clear unstained spots appeared at points where the enzymatic digestion took place. When digestion was partial, a clear ring appeared, since enzymatic action proceeded most rapidly at the rim of the drops. This allowed enzymatic digestion to be distinguished from the non-specific dissolution of the substrate film by control drops which sometimes caused a homogeneous faint decoloration of the film after a long period of incubation. The jelly film of *Rana japonica* prepared in the same way was found to be useful also as a substrate for the assay of *R. chensinensis* enzyme. However, the same enzyme failed to digest the jelly film of *Bufo bufo*. These results are consistent with the observed enzyme-substrate specificity which was demonstrated by placing fresh eggs of the heterologous species in hatching enzyme solutions (unpublished observation).

Because of the nature of the oviducal jelly material which tends to form irregular clumps during manipulation, it was not possible to prepare a perfectly uniform film. Thus the method may not be adaptable for an accurate quantitative assay. However, an approximate quantitative assay was possible, as will be presented later, by placing drops at an aimed interval of time on a film of the same dish, and choosing an arbitrary end point.

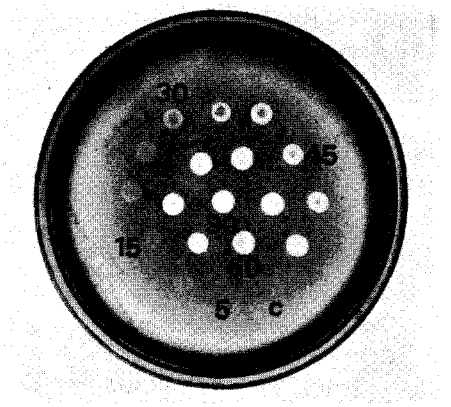

Fig. 1. Photograph of a jelly film plate which received drops of hatching enzyme solutions, showing digestion of the substrate film by the enzyme. Numbers in the figure indicate the time of incubation (in min). C, a control drop consisting of a buffer without enzyme. Stained with amido black. Explanation in text.

The approximate sensitivity of this assay system was tested in the following way: The outer jelly layers of St. 19 embryos were removed manually. The embryos were briefly immersed in 70% ethanol followed by repeated washings in sterilized water. The perivitelline fluid was collected with a fine glass pipette from a definite number of embryos and pooled. The volume of the fluid was adjusted to about 0.05 ml by evaporation at 1°C. When placed on the jelly film, it was found that the fluid collected from 15 embryos was sufficient to give a clear spot on a given jelly film after 2 hrs incubation at 37°C.

Some properties of hatching enzyme

The pH optimum of the hatching enzyme was determined as follows: A 67P fraction of the enzyme was dissolved in a potassium phosphate-borate buffer to a range of pHs. Drops of the enzyme solutions with different pHs were placed on the jelly film at 3 min intervals and incubated at 37°C, to find out the minimum period required for the digestion of the substrate film. As shown in Fig. 2, the optima for the enzyme are pH 7.4–7.8.

Since the preliminary observations suggested a higher enzyme activity in the 10%– than in the full strength Steinberg's solution, the effects of ions were tested with the jelly film method. The results summarized in Table 1 indicate that enzyme activity is affected by the specific cations in addition to the ionic strength. Additional experiments proved that the strong inhibition by divalent cations used was reversed by dialysis against deionized water, suggesting the utility of these ions for stabilization of the enzyme.

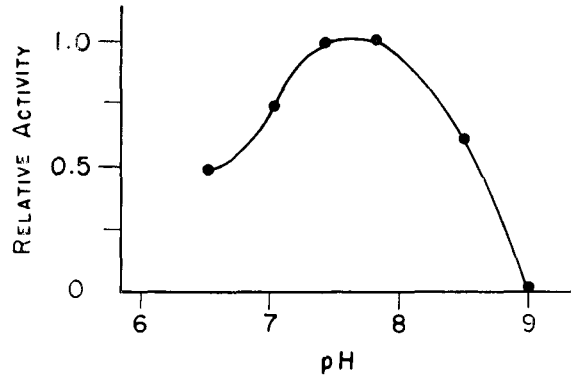

Fig. 2. The pH dependence of hatching enzyme activity as determined by the jelly film method.

Table 1. Effects of ions on the jelly digesting activity of hatching enzyme. Same concentration of enzyme was dissolved in various dilutions of Steinberg's solution or single salt solutions, and assayed by the jelly film method. Buffer, 0.05M Tris-HCl (pH 7.4); Temp. 37°C.

Enzyme dissolved in	Digestion of jelly film plate*		
	30 min.	60 min.	120 min.
1×Steinberg	−	±	+
1/2×Steinberg	−	+	+
1/4×Steinberg	−	+	+
1/8×Steinberg	±	+	+
1/10×Steinberg	±	+	+
M/16 NaCl	±	+	+
M/16 KCl	±	+	+
M/24 $CaCl_2$	−	−	±
M/24 $MgCl_2$	−	±	+
Deionized water	+	+	+

*+, Digested; ±, Partially digested; −, Undigested.

Table 2 summarizes the effects of inhibitors on the jelly film digesting activity of the enzyme. Apparent inhibition by IAA and PCMB may indicate the importance of the cysteine residue in the enzyme molecule. The failure of inhibition by SBTI is in agreement with the previously reported inability of trypsin to digest the egg-jelly (*cf.* Katagiri, 1963).

The jelly film assay system proved to be useful also in an attempt to follow the purification of the enzyme by gel-filtration. Fig. 3 depicts an elution profile of a crude enzyme which was filtered through a Sephadex G-100 column. From each fraction a drop of the sample was removed to place on the jelly film substrate.

Table 2. Effects of IAA, PCMB and SBTI on the hatching enzyme activity as determined by the jelly film method. Buffer, 0.05M Tris-HCl (pH 7.4); Temp. 37°C.

Reagent	Digestion of jelly film plate*		
	30 min.	60 min.	120 min.
Iodoacetamide	−	−	−
p-chloromercury benzoate	−	−	±
Soybean trypsin inhibitor	+	+	+
Control (None)	+	+	+

* +, Digested; ±, Partially digested; −, Undigested.

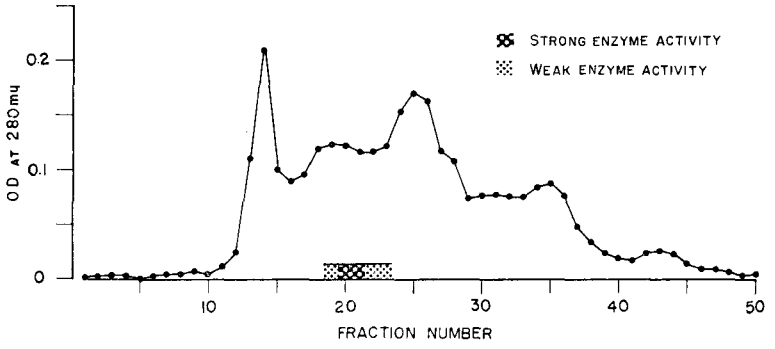

Fig. 3. Elution profile of a hatching enzyme preparation on Sephadex G–100 column. Fractions with the enzyme activity are also indicated. Bed dimensions, 2.5×41 cm; Eluant, 0.05 M Tris-HCl (pH 7.4); Flow rate, 5.5 ml/tube/15 min. Further explanation in text.

It was found that the spots from fractions 19–23 completely digested a given film upon 2 hrs incubation at 37°C. At 45 min incubation period the digestion was partial with these fractions except for fractions 20–21 which formed a clear spot. Apparently the enzyme was mostly concentrated in fractions 20 and 21. The confirmation of this result was obtained by an additional test in which 2 frozen blastulae were placed in aliquots from each of the fractions 18–30. Complete digestion of egg-envelopes occurred in fractions 20 and 21 after 4 hrs incubation at 37°C, whereas in the other fractions digestion was either confined to the outer layers of jelly, or was partial, or even negative after 24 hrs incubation. When applied on the same column under identical conditions, BSA and ovalbumin were eluted in fractions 19 and 23 respectively. Thus the approximate molecular weight of the enzyme concerned is 50,000–60,000.

Discussion

Compared with the other forms in which the hatching enzyme has been well studied, the amphibian enzyme has a rather unique feature in that it acts on both the vitelline membrane and the jelly envelopes, which are quite different in their biological origin and function as well as their physico-chemical natures. Methods for assessing enzyme activity using membranes of fresh eggs (sea urchins, Yasumasu, 1960) or fragments of chorions (teleosts, Kaighn, 1964; Yamagami, 1970) as a substrate would be applicable to the amphibian enzyme, too, at least as far as the vitelline membrane digesting activity is concerned. However, the unpublished observations to be presented elsewhere have demonstrated that the jelly and the vitelline membrane behave as the substrates which require a different order of specificity. Thus it should be stressed that the assay method developed in the present study can be applied to *only one* of the complex activities of the hatching enzyme.

The reliability of the present assay system has been demonstrated by a degree of the enzyme-substrate specificity which was parallel to results obtained when fresh egg envelopes were used as substrates. The use of the oviducal jelly film as a substrate has also been successful for preliminary characterization studies of the hatching enzyme from *Rana pipiens* (Katagiri, Kondo and Nace, unpublished). This method is basically similar to that described by Pickford and Dorris (1934), who employed gelatin surfaces of photographic lantern slide plates as a substrate for the small amount of digestive enzymes from spiders and amphibian embryos. Although essentially qualitative rather than quantitative, the simplicity of technique, the use of natural substrate, and the high sensitivity requiring an extremely small amount of enzyme solutions proved the usefulness of the present assay system, especially in following the enzymatic purification procedure. Attempts to develop an accurate quantitative method for assaying the jelly digesting activity have thus far been unsuccessful because of the inherent nature of the jelly, *viz.*, forming irregular clumps during manipulation, its unusually hydrophobic property, and the difficulty in precipitating it without structural change as an appropriate substrate for the enzyme, *etc.*

Results obtained by the present assay method do not explain the mode by which the substrate jelly is digested by the enzyme. In another series of experiments a quantitative determination of the proteolytic activity of the present enzyme has been successful with casein as a substrate (Katagiri, unpublished). Thus the combined use of these two assay methods has shown a striking resemblance in the enzymatic profiles of the proteolytic activity with the jelly-digesting activity presented above. Proteolytic hydrolysis of a number of synthetic substrates has also been demonstrated for the enzyme of *Xenopus laevis* (Carroll and Hedrick, 1972). In view of these results, together with the susceptibility of the jelly to a variety of proteolytic enzymes as evidenced earlier (Townes, 1953; Katagiri, 1963), it is quite possible to anticipate proteolysis as a major function of the hatching enzyme in destroying the integrity of the jelly. At present,

however, it seems premature to correlate these and other *in vitro* enzymatic activities with the observed biological activities of the enzyme before the molecular features of both the enzyme and the substrates in question are fully understood. In this respect the present assay system, in spite of its limitations, has the advantage of using a natural substrate in simplified form. Thus, with the aid of the present method, kinetics studies on enzyme production and secretion have now been possible with embryonic epidermal explants containing enzyme producing cells. The results will be published elsewhere.

Summary

A simple method was developed for detecting the jelly digesting activity of the hatching enzyme from the frog, *Rana chensinensis*. The method is based on placing and incubating a drop (0.03–0.05 ml) of the solution to be tested on dried film of the oviducal jelly which has been homogenized and layered at the bottom of a plastic petri-dish. Digestion by the enzyme resulted in the formation of a clear spot upon an appropriate staining of the substrate film. The sensitivity of the method was of the order that the enzymatic activity of perivitelline fluids collected from 15 pre-hatching tailbud embryos was detectable. Although essentially qualitative, the method could be adapted for the approximate quantitative assay of the enzyme activity.

The properties of the hatching enzyme as demonstrated by the present method were as follows: The pH optimum for the enzyme activity was between 7.4 and 7.8. The activity was significantly inhibited by Ca^{++}, Mg^{++}, iodoacetamide and *p*-chloromercuribenzoic acid, but was unaffected by a soybean trypsin inhibitor. The gel-filtration in combination with the enzymatic assay gave an estimation of 50,000–60,000 as a molecular weight of the enzyme concerned.

The validity of the assay method was discussed, with the emphasis on its convenience when only an extremely small amount of the sample is available.

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