Vol. 162, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 14, 1989 Pages 58-63

A UNIQUE PROTEOLYTIC ACTION OF HCE, A CONSTITUENT PROTEASE OF A FISH HATCHING ENZYME: TIGHT BINDING TO ITS NATURAL SUBSTRATE, EGG ENVELOPE

Shigeki YASUMASU, *Shigetaka KATOW, *Yukiko UMINO, Ichiro IUCHI and Kenjiro YAMAGAMI[1]

Life Science Institute, Sophia University, 7-1 Kioicho, Chiyoda-ku, Tokyo 102, Japan

*Department of Measles Virus, National Institute of Health, Musashimurayama, Tokyo 190-12, Japan

Received May 8, 1989

SUMMARY: High choriolytic enzyme (HCE), a constituent protease of the hatching enzyme of the teleost, Oryzias latipes, swells its natural substrate, egg envelope (chorion) by hydrolyzing it partially. This enzyme was found to be bound tightly to the chorion when it exerted catalytic action. This was evidenced by the experimental results showing (i) that the turnover of this enzyme seemed to be hindered by the chorion, (ii) that the enzyme bound to the chorion could be recovered by washing with an alkaline medium, and (iii) that the bound enzyme could be quantified by radioimmunological estimation. The bound enzyme sustained its original activity and the binding between the enzyme and the chorion seems to be stoichiometric.

The hatching enzyme is secreted by the animal embryo at the time of hatching and participates in solubilization of the egg envelope which encases the embryo and protects it from harm during development. Recently, it was found in the teleost, Oryzias latipes, that the hatching enzyme was not a single enzyme but an enzyme system composed of two distinct zinc proteases, high choriolytic (egg envelope digesting) enzyme (HCE) and low choriolytic enzyme (LCE) (1-3). They solubilize a hard egg envelope (chorion) by acting cooperatively: HCE swells the inner layer of chorion with concomitant release of some peptides from it and LCE solubilizes efficiently only the swollen portion of the chorion, leaving a thin fragile sheet of undigestible outer layer of the chorion. In the course of studies on the mechanism of their choriolytic action, we found that HCE was strongly bound to the chorion when

[1] To whom correspondence should be addressed.

0006-291X/89 $1.50

mixed with this natural substrate. The tight binding of HCE to the chorion seems to be prerequisite to its action of choriolytic swelling. In this report, we describe some experimental results showing the tight binding of the enzyme to its substrate.

MATERIALS AND METHODS

Materials: High choriolytic enzyme (HCE) was purified from the hatching liquid of the cultured Oryzias latipes embryos according to the procedure described in a previous paper (2). The natural substrate for this enzyme, the egg envelope (chorion), was isolated by crushing blastulae with a loosely fitted Teflon homogenizer as described previously (4).

HCE assays: HCE activity was determined using the chorion by two different methods: The turbidimetric method and the densitometric method. The turbidimetric method was based on the measurement of the increase in transmission at 610 nm of the Tris·HCl buffered reaction mixture (pH 7.5) containing chorion paste suspension as substrate (see 4). The activity was expressed in terms of the percent increase of the transmission during the initial one minute ($\triangle T_{610}$%/min)(1,2). The densitometric method measures the amount of peptides released from coarse fragments of chorion in terms of the increase in absorbance at 280 nm of the supernatant of the reaction mixture (2). As reported in the previous paper (2), the chorion fragments were markedly swollen during the peptide release and there was a proportional relationship between these two phenomena. Thus, this action of HCE can be named choriolytic swelling. The swollen chorion, however, remained insoluble for a long time even in an excess amount of HCE, as HCE hardly digests the swollen portion of the chorion (2).

"Washing" experiment: In order to analyze the interaction between HCE and the chorion, "washing" experiments were performed. Twenty milligrams of coarse fragments of chorion were mixed with 6, 12 or 18 μg of the purified HCE and incubated in 50 mM Tris·HCl-10 mM NaCl (pH 7.5) at 30℃ for 30 min. The supernatant (spnt I) was separated from the chorion fragments. The chorion fragments were then washed with 20 ml of distilled water three times, suspended in 1 ml of either 50 mM Tris·HCl buffer (pH 7.5) or 50 mM bicarbonate buffer (pH 10.2) and incubated with continuous shaking at 30℃ for 10 min. The supernatant (spnt $II_{7.5}$ or spnt $II_{10.2}$, respectively) was again separated from the chorion fragments. This "washing" procedure was repeated two times and the washings were combined. The HCE activity in the supernatants I and II was determined by turbidimetry. In the control, the same amounts of the purified HCE were incubated without 20 mg of chorion fragments.

Preparation of monoclonal anti-HCE antibody (MAB): MAB was raised against the purified HCE in Balb/c mice according to the procedure described elsewhere (5). IgG fraction was obtained from the ascites of a Balb/c mouse injected with hybridoma cells, and Fab' of MAB was prepared.

Radioimmunological estimation of HCE bound to chorion: The amount of HCE bound to chorion was quantified radioimmunologically. Two milligrams of coarse chorion fragments were incubated with varied amounts of HCE in 0.1 ml of a mixture composed of 20 mM Tris·HCl-50 mM NaCl (pH 7.5), 0.5 % bovine serum albumin and 0.05 % Tween 20 at 0℃ for 5 min. This buffer mixture was also employed as a diluent or washing medium to prevent non-specific binding of HCE and MAB Fab' to the chorion. Under these conditions, the chorion fragments were not swollen by the action of HCE. After the chorion fragments were washed with 5 ml of the buffer three times, they were reacted with 12 μg

of Fab' of MAB (A-33) in 0.3 ml of the same buffer at 4℃ for 1 hr. The unbound Fab' was removed by washing with 5 ml of the buffer three times. The chorion fragments thus treated were then reacted with [^{125}I]F(ab')$_2$ of rabbit anti-mouse IgG antibody (1 μCi/0.3 ml, Amersham Int. plc, Buckingham) at 4℃ for 1 hr. After washing with 5 ml of the buffer four times, the radioactivity of 0.3 ml of the chorion suspension was counted in a gamma counter.

RESULTS AND DISCUSSION

When a definite amount of coarse fragments of chorion was incubated with varied amounts of the purified HCE, a peculiar set of time-course curves for choriolytic swelling was obtained. Figure 1 shows the result obtained when 20 mg of chorion fragments were incubated with 2.0, 5.0 or 12.0 μg of HCE at 30℃ for 90 min. The choriolytic swelling was determined by the densitometric method. During the initial 30 min, the choriolytic swelling proceeded at a velocity approximately corresponding to the amount of the enzyme added. After 30 min of incubation, however, the velocity fell remarkably regardless of the amount of the added HCE, and each curve leveled off. It was found that 12 μg of HCE was sufficient to cause a full swelling of 20 mg of chorion fragments, as the time-course curve with more than 12 μg of HCE also became flat at the same level as that with 12 μg of HCE (Fig. 2). As reported previously (2), HCE hardly attacked further the swollen chorion. Even when less than 12 μg of HCE was added, the velocity of choriolytic swelling fell

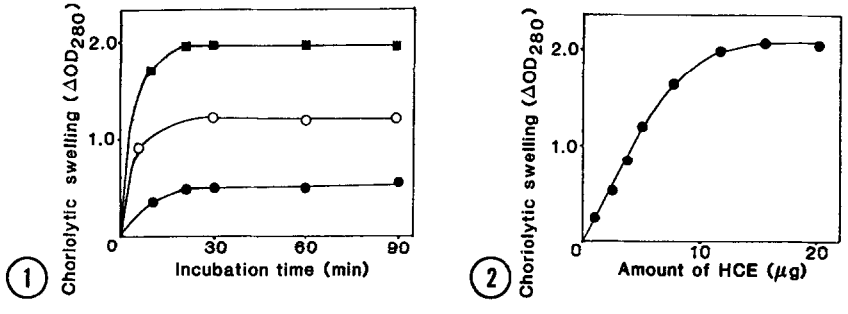

Figure 1. Time course of choriolytic swelling of the egg envelope (chorion) by the purified HCE of Oryzias latipes. Twenty milligrams of coarse fragments of intact chorion were incubated with various amounts of HCE in 1 ml of the reaction mixture consisting of 50 mM Tris·HCl-10 mM NaCl (pH 7.5) at 30℃ with continuous shaking. Choriolytic swelling is expressed in terms of the increase in absorbance at 280 nm of the supernatant of the reaction mixture. The curves with 2(●), 5(○) and 12 (■) μg of HCE are presented.

Figure 2. Relationship between the choriolytic swelling and the amount of HCE. The values of choriolytic swelling after 30 min-incubation were plotted against the amount of the HCE used.

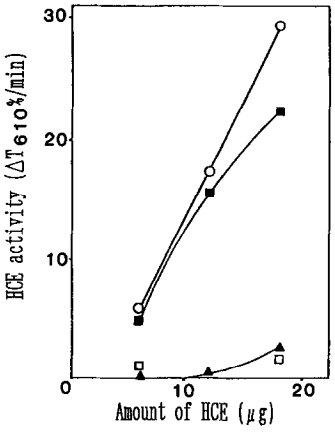

Figure 3. Interaction between HCE and the chorion of Oryzias latipes. Twenty milligrams of coarse fragments of chorion were mixed with 6, 12 or 18 μg of HCE and incubated in 50 mM Tris·HCl-10 mM NaCl (pH 7.5) at 30°C for 30 min. After the supernatant (spnt I) was separated from the chorion, the swollen chorion fragments were washed with distilled water two times, followed by incubation with 1 ml of neutral (pH 7.5) or alkaline (pH 10.2) medium. HCE activity in spnt I (▲), spnt $II_{7.5}$ (□) or spnt $II_{10.2}$ (■) was determined by turbidimetry, as described in MATERIALS AND METHODS. Control (○) refers to the activity of the same amounts of HCE added into the incubation mixture containing no chorion fragments.

remarkably after 30 min despite there being enough substrate still remaining intact. This peculiar phenomenon suggested that the turnover of HCE was hindered probably because HCE might be either inactivated once it has acted on the chorion or bound tightly to the chorion.

To determine which of these alternatives was the case for HCE, "washing" experiments were performed. As shown in Figure 3, the HCE activity was scarcely found in the supernatant I when less than 12 μg of HCE was mixed with 20 mg of chorion. If more than 12 μg of HCE was added, some activity was found in the supernatant I. On the other hand, most of the activity was found in the supernatant $II_{10.2}$, while no significant activity was in the supernatant $II_{7.5}$. From these results, it was strongly suggested that HCE was not inactivated but bound to the chorion fragments and that the bound enzyme was not easily released on incubation in a neutral medium but released in an alkaline medium. The result of Figure 3 also indicated that 20 mg of chorion was saturated with approximately 12 μg of HCE. This seems to be compatible with the results presented in Figures 1 and 2.

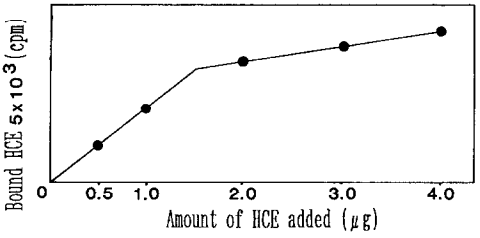

Figure 4. Radioimmunological estimation of the amount of HCE bound to the chorion. Two milligrams of coarse fragments of chorion were incubated with varied amounts of HCE in 0.1 ml of a mixture composed of 20 mM Tris·HCl-50 mM NaCl (pH 7.5), 0.5 % bovine serum albumin and 0.05 % Tween 20 at 0℃ for 5 min. The amount of HCE was determined by using Fab' of monoclonal anti-HCE antibody (A-33) and [^{125}I]F(ab')$_2$ of rabbit anti-mouse IgG antibody as probes, as described in MATERIALS AND METHODS.

The result of the radioimmunological estimation of the bound HCE to the chorion is shown in Figure 4. It was found that the amount of HCE bound to 2 mg of chorion fragments increased linearly up to about 1.5 μg but the increasing rate was markedly reduced thereafter. These results are again in accordance with the above-described results that 20 mg of chorion was saturated with about 12 μg of HCE. Thus, the binding of HCE to the chorion seems to be stoichiometric. The present results showed that HCE was, unlike ordinary enzymes, bound tightly to its substrate, the chorion, when it exerted the action of choriolytic swelling and that the enzyme remained bound to the swollen chorion. Although such behavior of HCE is apparently very similar to that of the non-enzymic vitelline coat lysins from the sperm of some gastropods (6-8), the mechanism remains unsolved at present. As reported previously (2), HCE hydrolyzes casein besides the chorion probably through the same catalytic site. We have obtained another monoclonal anti-HCE antibody (MAB, E-72) and found that this MAB inhibited the HCE activity of both the choriolytic swelling and the binding to the chorion, while it did not affect the caseinolytic activity. These results suggest that the binding of HCE to the chorion is closely related to its choriolytic swelling activity and that HCE has a binding site which is independent of the catalytic site on its molecule (Yasumasu et al., unpublished). It seems likely that such a tight binding of HCE to its substrate is a special attribute of this enzyme to facilitate solubilization of a hard biological structure such as egg envelope.

ACKNOWLEDGMENTS

The authors wish to thank Dr. J. M. Michalec for reading through the manuscript. This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

REFERENCES

1. Yasumasu, S., Iuchi, I. and Yamagami, K. (1988) Zool. Sci., 5: 191-195
2. Yasumasu, S., Iuchi, I. and Yamagami, K. (1989) J. Biochem., 105: 204-211
3. Yasumasu, S., Iuchi, I. and Yamagami, K. (1989) J. Biochem., 105: 212-218
4. Yamagami, K. (1970) Annot. Zool. Japon., 43: 1-9
5. Kennett, R. H. (1980) In Monoclonal Antibodies., Ed. by R. H. Kennet, T. J. McKearn and K. B. Bechtol. Plenum Press N. Y.
6. Haino, K. (1971) Biochim. Biophys. Acta., 229: 459-470
7. Haino-Fukushima, K. (1974) Biochim. Biophys. Acta., 352: 179-191
8. Ogawa, A. and Haino-Fukushima, K. (1984) Dev. Growth Diff., 26; 345-360