ELSEVIER

FEBS Letters 339 (1994) 281–284

FEBS 13680

FEBS LETTERS

HCE, a constituent of the hatching enzymes of *Oryzias latipes* embryos, releases unique proline-rich polypeptides from its natural substrate, the hardened chorion

Kyong-Su Lee[a,**], Shigeki Yasumasu[a], Kohji Nomura[b], Ichiro Iuchi[a,*]

[a]*Life Science Institute, Sophia University, 7–1 Kioi-cho, Chiyoda-ku, Tokyo 102, Japan*
[b]*Department of Biochemistry, Tokyo Metropolitan Institute of Gerontology, 35–2 Sakae-cho, Itabashi-ku, Tokyo 173, Japan*

Received 6 December 1993; revised version received 12 January 1994

Abstract

HCE, a constituent protease of the hatching enzymes of *Oryzias latipes* embryos [1,2], releases unique proline-rich polypeptides from its natural substrate, the hardened chorion. The polypeptides consist of repeats of Pro-X-Y, mainly Pro-Glx-X. In addition, the polypeptides contain abundant γ-glutamyl ε-lysine isopeptides which are regarded to be responsible for chorion hardening. These findings suggest that HCE recognizes specific site(s) of the chorion, releases the proline-rich polypeptides from it, and makes the substrate accessible to LCE, another protease of the hatching enzymes.

Key words: Hatching enzyme; HCE; *Oryzias latipes*; Pro-X-Y repeat; γ-Glutamyl ε-lysine crosslink

1. Introduction

Hatching enzyme system of *Oryzias latipes* embryos consists of two zinc metalloproteases; HCE, high choriolytic enzyme and LCE, low choriolytic enzyme [1]. Characterization of cDNAs for them showed that both enzymes consisted of 200 amino acids containing the common His-Glu-X-Y-His motif for Zn protease and belonged to the astacin (protease) family. The similarity between HCE and LCE was 55% [3].

HCE swells its substrate, the inner layer of the hardened chorion, by its partial proteolytic action, while LCE cannot digest the intact chorion but can digest completely the swollen chorions [2,4,5]. Therefore, the proteolytic action of HCE to the chorion is prerequisite for the initiation of the chorion-digesting process by the hatching enzyme system.

HCE is a unique enzyme with regard to its proteolytic action on the chorion. Kinetic studies [6] and binding experiments [7] have shown that HCE tightly binds to the chorion prior to partial digestion and swelling of it. The fact that HCE binds to the hardened chorion has led us to investigate the possibility that HCE recognizes and binds to specific site(s) on the hardened chorion, and cleaves and releases specific polypeptides or peptides. Accordingly, we examined some characteristics of the peptides released by HCE from the hardened chorion.

2. Materials and methods

HCE was purified from the hatching liquid, the culture medium of the hatching embryos of *Oryzias latipes*, according to the method reported previously [2,8]. Hardened chorions, the natural substrate of HCE, were isolated from blastulae, washed, fragmented using a blender, and lyophilized.

When HCE was incubated with the hardened chorion, the time course of its choriolytic action was characterized by biphasic change [6]. HCE elicits swelling of the chorion with concomitant release of peptides from it (initial phase). When the chorion is maximally swollen, the rate of the reaction slows down profoundly to about 1% of the initial rate (final phase). In the initial phase of chorion swelling, there is a stoichiometric relationship between HCE and its substrate, the hardened chorion [6]. Twenty mg of the isolated and lyophilized chorion fragments were incubated with 12 μg of HCE in 1 ml of 10 mM NaCl, 20 mM Tris-HCl (pH 7.5) at 30°C for 30 min. The reaction was stopped by adding ethylenediaminetetraacetic acid (final concentration, 1 mM). This reaction corresponds to the initial phase of chorion digestion. The supernatant was obtained by centrifuging the reaction mixture at 18,500 × *g* for 30 min and subjected to various analyses.

Chorion digests thus prepared were fractionated through gel filtration chromatography using a Toyopearl HW-50SF (1.5 × 89 cm; TOSOH Inc., Tokyo) column equilibrated with 150 mM NaCl, 100 mM Tris-HCl (pH 7.8). The elution was performed at a flow rate of 30 ml/h and monitored by absorption at 280 nm. Peak H1 and Peak H2 were obtained as shown in Fig. 1.

Peak H2 polypeptides were further fractionated by reverse-phase chromatography in a HPLC system using a Shodex C8–5A column (4.6 × 150 mm; Showadenko Co.). The fractionation was performed with a linear gradient from 0 to 90% acetonitorile in 0.1% trifluoroacetic acid (pH ca. 2) at a flow rate of 1.0 ml/min. The elution was monitored by absorption at 215 nm. This procedure was repeated several times until a fraction of a sharp and single peak was obtained (Fig. 2). Each of the fractions, a, b or c, thus isolated from Peak H2

*Corresponding author. Fax: (81) (3) 3238 3885.

**Present address:* Laboratory of Cell and Developmental Biology, Faculty of Biosciences, Tokyo Institute of Technology, Nagatsuta, Midori-ku, Yokohama 227, Japan.

SSDI 0014-5793(94)00064-3

was dotted on polyvinyliden difluoride membrane and subjected to sequence analysis using a Shimadzu protein sequencer PSQ-1.

Estimation of γ-glutamyl ε-lysine isopeptide contents was carried out essentially according to the method previously reported [9,10]. Peak H1, Peak H2 or whole chorion was digested by Pronase E (200 μg/day, total 800 μg; Sigma Chemical Co.) in 5 mM $CaCl_2$, 50 mM Tris-HCl (pH 7.5) for 4 days at 37°C and finally, the reaction medium was boiled for 10 min to inactivate the Pronase E. After $MgCl_2$ (final concentration, 5 mM) was added to the reaction medium, further proteolytic digestion was performed by the sequential treatment of leucine aminopeptidase (560 μg; Sigma Chemical Co.) for 19 h, carboxypeptidase Y (200 μg; Sigma Chemical Co.) for 12 h, prolidase (100 μg; Sigma Chemical Co.) for 12 h and leucine aminopeptidase (280 μg) for 12 h. All procedures were performed at 37°C in the presence of a trace of thymol. The sample thus digested was subjected to an amino acid analyzer JEOL JLC-300 (Nihon Denshi Co.) to estimate the γ-glutamyl ε-lysine contents.

Amino acid analysis of the samples hydrolyzed by 6 N HCl was performed according to the ordinary method using a Hitachi amino acid analyzer L-8500 at Toray Research Center Inc., Tokyo.

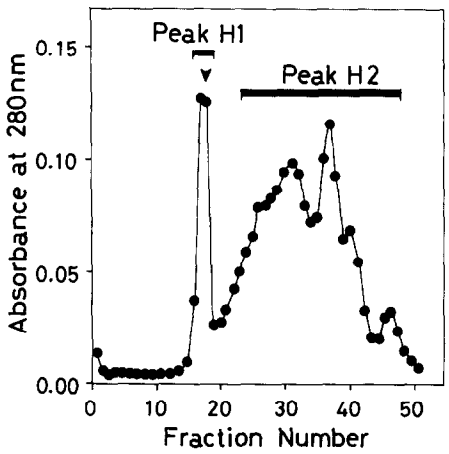

Fig. 1. Fractionation of an HCE-digest of the hardened chorion by gel filtration. The arrowhead indicates the void volume of the column.

3. Results

HCE swells the hardened chorion by partial proteolysis, releasing a significant amount of peptides [6]. We have identified the molecular structure of some of the released peptides.

As shown in Fig. 1, gel filtration chromatography separated the released polypeptides into two fractions; one was a sharp peak fraction eluted at the void volume of Toyopearl HW-50SF column (Peak H1) and the other was a broad peak fraction (Peak H2), the molecular weights of which ranged from 10,000 to 50,000. The appearance of two such peaks is similar to the previous result of gel filtration chromatography of hatching liquid [11].

Table 1
Amino acid composition of whole chorion, Peak H1 or Peak H2

Amino acid	Whole chorion (mol%)	Peak H1 (mol%)	Peak H2 (mol%)
Lysine	4.51	3.77	7.30
Histidine	2.18	2.65	1.21
Arginine	3.13	3.68	0.46
Aspartic Acid	9.26	9.18	9.16
Threonine	4.78	6.00	1.78
Serine	7.11	6.69	6.57
Glutamic Acid	14.67	9.77	24.34
Proline	16.42	9.53	33.28
Glycine	5.60	6.45	2.21
Alanine	5.73	7.73	0.59
Cystine/2	1.49	4.36	0.00
Valine	6.50	7.84	3.62
Methionine	0.75	1.06	0.06
Isoleucine	3.11	3.86	0.09
Leucine	5.77	7.84	0.50
Tyrosine	5.68	4.64	8.41
Phenylalanine	3.31	4.51	0.43
Tryptophan	ND	0.45	0.00

Data of amino acid composition of whole chorion was cited from the paper of Iuchi and Yamagami [11]. The composition of tryptophan in whole chorion was not determined (ND).

We compared the amino acid composition between the whole chorion, peak H1, and peak H2. As shown in Table 1, Peak H2 had a unique amino acid composition; a very high content of proline (33.3 mol%) and glutamic acid/glutamine (24.3 mol%), a relatively high content of lysine (7.30 mol%) and tyrosine (8.41 mol%), and a low content of arginine, threonine, alanine, glycine, isoleucine, leucine and phenylalanine. The uniqueness of Peak H2 polypeptides was also represented by the finding that their bands on SDS-PAGE were hardly stained with Coomassie brilliant blue R-250, and similarly not by the silver staining method nor the copper staining method. The finding strongly suggests that Peak H2 consists of unique proline-rich polypeptides. The characteristic of relatively high contents of proline (16.42 mol%) and glutamic acid/glutamine (14.67 mol%) in the whole chorion is ascribable to its inclusion of such proline- and glutamic acid/glutamine-rich peptides.

The chorion of unfertilized eggs is soft and fragile. After fertilization, the chorion becomes hardened and protects the embryos from the mechanical stress. This chorion hardening has been suggested to be due to a formation of γ-glutamyl ε-lysine crosslinks between constituent proteins of the chorion [9,12,13]. At the hatching of the embryos, the hatching enzymes digest this hardened chorion. Therefore, we compared the content of γ-glutamyl ε-lysine isopeptides between whole chorion, Peak H1 and Peak H2. As shown in Table 2, the isopeptide content in Peak H2 was found to the 4-fold and 10-fold higher than those in the whole chorion and Peak H1, respectively. Thus, most of the γ-glutamyl ε-lysine cross-links in the whole chorion are localized to the Peak H2 polypeptides.

We isolated several fractions of polypeptides from Peak H2 as sharp peaks by the repeated reverse-phase chromatography (Fig. 2) and determined amino acid sequences of three of them from their N-termini (Peak H2 a, b and c). As shown in Fig. 3, the N-terminal 6–7 amino

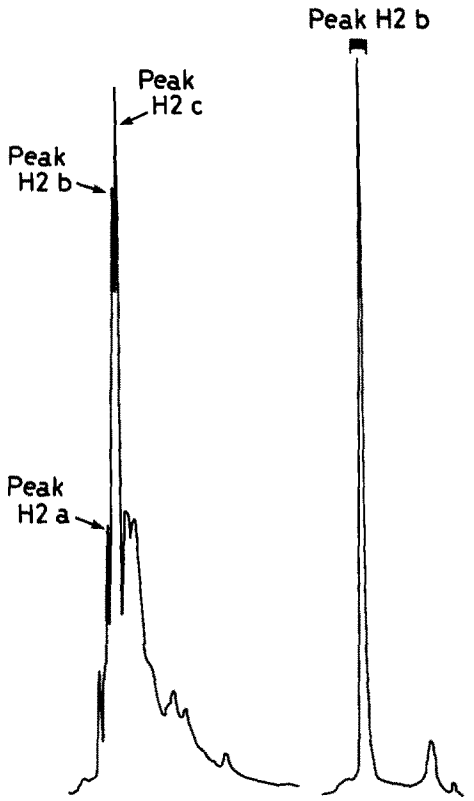

Fig. 2. Fractionation of Peak H2 by reverse-phase HPLC. Left profile indicates the fractionation of whole Peak H2. Peak H2 a, b or c was fractionated repeatedly several times by the same method until a sharp and single peak was obtained. As an example, the profile of Peak H2 b isolated finally is indicated on the right.

acid residues of them were composed of a limited selection of amino acids. The amino acid sequences were characterized as follows: first, the N-terminal amino acid in the majority of peptides was tyrosine, and aspartic acid or asparagine in a minority of peptides. Second, the second and fifth amino acids from the N-terminus were exclusively proline. Third, the third and sixth were glutamine as a major constituent and glutamic acid or serine as a minor one. Finally, the forth was mostly lysine or valine. These findings suggest that the proline-rich and γ-glutamyl ε-lysine-rich Peak H2 polypeptides contain some Pro-X-Y repeats, the more precise structure of which is mainly Pro-Glx-X.

Table 2
Content of γ-glutamyl ε-lysine isopeptide (γ-Glu ε-Lys) of whole chorion, Peak H1 or Peak H2

	Whole chorion (nmol/mg protein)	Peak H1 (nmol/mg protein)	Peak H2 (nmol/mg protein)
γ-Glu ε-Lys	63.6	23.4	273

4. Discussion

When HCE swells the hardened chorion during the initial phase of its proteolytic action, this enzyme releases various kinds of polypeptides from it. Among them, Peak H2 polypeptides had unique characteristics; especially, the contents of proline, glutamic acid/glutamine, and γ-glutamyl ε-lysine isopeptide were higher than whole chorion or Peak H1.

It has been reported that high molecular weight proteins (F1 and F2) were isolated from the digest of chorions by Sephadex G-200 [11]. The hatching enzyme sample used in that study is considered to contain both HCE and LCE [5]. The amino acid compositions of them are essentially similar to those of Peak H1.

Peak H2 was composed of various kinds of polypeptides such as Peak H2 a, b and c. Some peptides other than Peak H2 a, b, and c were also isolated as single peaks and sequenced by the same method (data not shown). They had characteristic amino acid sequences common with Peak H2 a, b, and c; that is, the 2nd and 5th positions in N-teminal 6–7 amino acid residues are exclusively proline, and the 3rd and 6th are highly probably glutamine. This similarity among them suggests that almost all the peptides contained in Peak H2 consist commonly of Pro-X-Y repeats where X is Gln or Glu.

Peak H2 a, b, or c, even though obtained as a sharp and single peak by repeated reverse-phase HPLC, is considered to contain heterogeneous N-termini mainly because of γ-glutamyl ε-lysine crosslinks. In addition, we could not identify amino acids along the whole length of their sequences because the cycles of Edman degradation could not proceed over 6 or 7 amino acids from

```
Peak H2 a
  N- Tyr - Pro - Gln - Lys - Pro - Gln - Asn -
    (Asn)      (Ser) (Asn)      (Glu) (Asp)
    (Asp)      (Glu)
    (Ala)

Peak H2 b
  N- Asn - Pro - Gln - Val - Pro - Gln -
    (Tyr)      (Glu) (Tyr)
    (Asp)

Peak H2 c
  N- Tyr - Pro - Gln - Lys - Pro - Gln -
    (Asn)      (Ser) (Val)
    (Asp)      (Glu)
```

Fig. 3. Amino acid sequences of some polypeptides purified from Peak H2 by reverse-phase HPLC. Major amino acid identified in each cycle of Edman degradation was arranged from N-terminus at the upper part and minor one was at the lower part as indicated in parentheses. Amino acid appearing in 2nd and 5th cycles was exclusively proline.

N-termini. However, one third of total mols of amino acids in Peak H2 polypeptides was proline and the incidence of amino acids in Peak H2 polypeptides as a whole was almost identical to that of amino acids observed by sequencing Peak H2 a, b, and c. Therefore, it is conjectured that Pro-X-Y, probably Pro-Glx-X, repeats are uniformly distributed along almost all the length of any Peak H2 polypeptide.

HCE itself could not cleave the γ-glutamyl ε-lysine isopeptide bonds responsible for the chorion hardening (data not shown). It would digest the surroundings of them and bring about the chorion-swelling probably because of exposure of hydrophilic regions. In addition, the chorion-swelling action of HCE would probably expose some sites which are sequestered in the intact hardened chorion and are susceptible to LCE attack. In fact, LCE can easily digest soft chorion of the unfertilized egg, which contains a lower quantity of γ-glutamyl ε-lysine isopeptides than hardened chorion of the fertilized egg (unpublished data).

In conclusion, although the precise molecular mechanism of interaction between HCE and the chorion is not yet known, it is possible that HCE recognizes and cleaves specific sites of the hardened chorion, and releases unique proline-rich polypeptides such as Peak H2. The specific digestion by HCE results in making a substrate accessible to LCE.

Acknowledgements. We wish to express our cordial thanks to Prof. K. Yamagami in Sophia University, Tokyo, for supporting the present study, giving us valuable advice throughout the study, and reading this manuscript.

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., Yamada, K., Akasaka, K., Mitsunaga, K., Iuchi, I., Shimada, H. and Yamagami, K. (1992) Dev. Biol. 153, 250–258.
[4] Yasumasu, S., Iuchi, I. and Yamagami, K. (1989) J. Biochem. 105, 212–218.
[5] Yamagami, K., Hamazaki, T.S., Yasumasu, S., Masuda, K. and Iuchi, I. (1992) Int. Rev. Cytol. 136, 51–92.
[6] Yasumasu, S., Katow, S., Umino, Y., Iuchi, I. and Yamagami, K. (1989) Biochem. Biophys. Res. Commun. 162, 58–63.
[7] Lee K.S., Yasumasu, S., Iuchi, I. and Yamagami, K. (1992) Zool. Sci. 9, 1157.
[8] Yamagami, K. (1972) Dev. Biol. 29, 343–348.
[9] Hagenmaier, H.E., Schmitz, I. and Foehles, J. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 1435–1438.
[10] Nomura, K. and Suzuki, N. (1991) in: Proceeding of the 7th International Echinoderm Conference, Biology of Echinodermata, 283–289.
[11] Iuchi, I. and Yamagami, K. (1976) Biochim. Biophys. Acta 453, 240–249.
[12] Oppen-Berntsen, D.O., Hervik, J.V. and Walther, B.T. (1990) Dev. Biol. 137, 258–265.
[13] Masuda, K., Iuchi, I. and Yamagami, K. (1991) Dev. Growth Differ. 33, 75–83.