

# Mechanical Analysis of Chorion Softening in Prehatching Stages of Zebrafish Embryos

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**Abstract**—During early development, the chorion envelope of the zebrafish embryo undergoes a thinning process called “chorion softening,” which has so far only been characterized chemically. In this study, a micromechanical force sensing system was used to characterize and quantitate mechanical modifications of the zebrafish embryo chorion during early development. Quantitative relationships between applied forces and chorion structural deformations were established at various embryonic stages. The measured penetration force into the chorion at the blastula stage was 1.3-fold greater than those at the prehatching stage. Furthermore, chorion elastic modulus values were determined by using a biomembrane elastic model. The elastic modulus of the chorion at the blastula stage was 1.66-fold greater than that at the prehatching stage, thus indicating that the chorion envelope become mechanically “softened” at the prehatching stage. The experimental results quantitatively describe “chorion softening,” which is most likely due to proteolytic activities at the prehatching stage. Gradual chorion softening during embryonic development was also artificially achieved by treating blastula chorion with pronase, a proteolytic enzyme. The forces required to penetrate the pronase-treated chorion were similar to those at the prehatching stage. This similarity suggests that “chorion softening” may be induced by the release of protease from the embryos, and the chemical nature of the process involves proteolytic fragmentation of the ZP2 protein.

**Index Terms**—Cellular force sensing, chorion softening, elastic modulus, hatching, peptide fragmentation, protease, proteolytic activities, zebrafish embryo.

## I. INTRODUCTION

THE relationship between mechanical forces and cellular deformations in living organisms is important for understanding a variety of cell functions. Several methods have been

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developed for mechanically characterizing cell membranes including the extracellular matrix (ECM) [1]–[7]. A tensile test system has been designed and applied to fibroblasts from the rabbit patellar tendon [1]. An effective instrument embodying a video enhanced microscope and a theoretical linear elastic solution were developed to characterize the elastic modulus of a thin biological membrane [2]–[4]. The mechanical behavior of living cells was studied with micropipette suction in which the surface of a cell is aspirated into a capillary pipette while tracking the leading edge of its surface [5]. Microelectromechanical systems (MEMS)-based cellular force sensors were developed to quantitate mechanical property changes of the mouse zona pellucida (ZP) during fertilization [6] or measure forces generated by living heart muscle cells [7]. Despite these efforts, there has been no quantitative study on the chorion, which is the ECM of zebrafish embryos.

The zebrafish is a recently emerging model organism for addressing questions of vertebrate embryo development [8]–[10]. The chorion envelope of zebrafish embryos undergoes a thinning process called “chorion softening” before the basic body formation is complete and hatching occurs [11]. Upon hatching, the chorion is digested by hatching enzymes that are proteolytic enzymes secreted from hatching gland cells of the embryo [12], [13]. The hatching gland cells of zebrafish are in the epidermis of the yolk sac and are originated from the anterior end of the hypoblast, the polster [14]. The hatching enzymes have been partially identified, which may be responsible for “chorion softening” [15].

Similar to chemically characterizing “zona hardening” [16]–[18], biological studies have employed chemicals to test the period for dissolving the chorion, which chemically rather than mechanically describes hardness/softness of the chorion. For example, proteases, such as high choriolytic enzyme (HCE) and cathepsin L, that are a constituent protease of the hatching enzyme were shown to bind tightly to the chorion as well as swell the chorion by partially hydrolyzing it [8], [19]. The chorion envelope undergoes chemical modifications during both fertilization and hatching for successful embryonic development. It was speculated that these chemical modifications might cause mechanical changes.

The zebrafish is an attractive model for manipulations and studies of biophysical properties due to the relatively large egg size and the fact that basic cellular processes (i.e., fertilization, embryonic development, and hatching) involving the ECM are well-conserved among vertebrates. Therefore, an understanding of mechanical modifications of zebrafish egg ECM will allow for obtaining insights into the clinical problems arising in

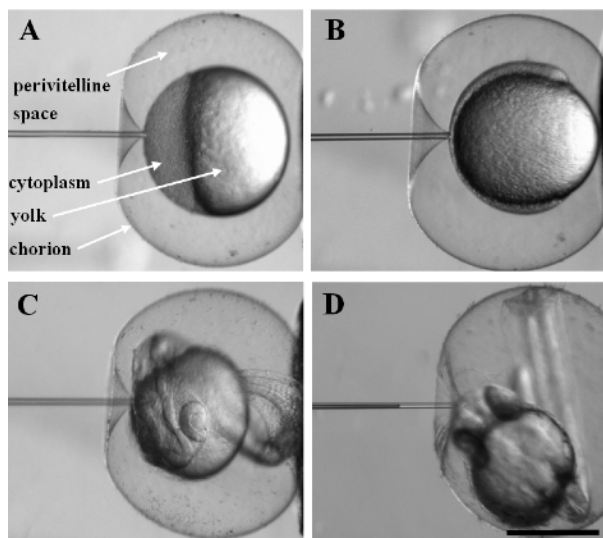


Fig. 1. Zebrafish embryos at different developmental stages. Pipette angle in the loading experiments is carefully controlled so that the pipette is loaded perpendicularly to the chorion. Blastula (A, 3 hpf); gastrula (B, 7.5 hpf); pharyngula (C, 24 hpf); and pre-hatching stage (D, 36 hpf). Scale bar is 200  $\mu\text{m}$ .

polyspermy and implantation failure due to inappropriate ECM hardening and incomplete hatching. For example, one of the zebrafish chorion components called ZPC protein gene has been cloned and identified as a homologous zebrafish gene product to a mouse ZP protein, called ZP3. Upon fertilization, the zebrafish chorion undergoes chemical modifications similar to mouse ZP proteins during fertilization [20].

In order to quantitate the mechanical modifications of the chorion during early development, a micromechanical force sensing system was developed in this study. Quantitative relationships between applied forces and chorion structural deformations were established for various developmental stages. The gradual decrease of the measured chorion penetration forces from blastula to pharyngula stages was closely associated with a well-developed hatching gland that is present at the pre-hatching stage on the pericardium over the anterior yolk sac, secreting proteases to dissolve the chorion at the end of the embryonic development [21]. The chemical nature of the softening process may be due to the fragmentation of the ZP2 protein that is caused by the proteolytic enzymes released from hatching embryos.

## II. METHODS

### A. Embryo Preparation

For force-deformation measurements in developing embryos, ten embryos were collected from each typical developmental stage (Fig. 1). Embryos were accurately staged by examining morphological features of each living embryo under the microscope according to the standard stages of embryonic development of the zebrafish [21]. Each stage was clearly discriminated due to the transparent cell structure. Following the blastula stage, embryos develop into the gastrula stage at 7.5 h postfertilization (hpf) with three germ layers formed. The embryo, called pharyngula (prim-5) at 26 hpf, shows distinct movement within the chorion. Between 42 and 48 hpf, the stage is known as pre-hatching (high-pec). The embryo hatches

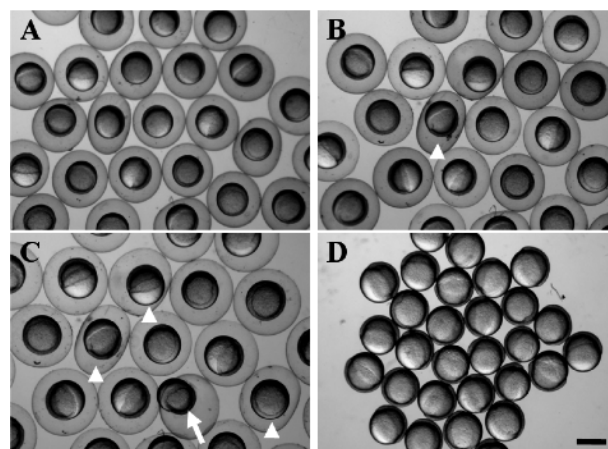


Fig. 2. Pronase induced artificial "chorion softening." embryos at the blastula stage were incubated for (a) 0, (b) 10, (c) 15, and (d) 40 min in pronase-containing ZCM. Note that the chorions of some embryos are swelling [arrowheads in (b) and (c)], hatching [an arrow in (c)] or denuded (d) due to the proteolytic enzyme. Scale bar is 300  $\mu\text{m}$ .

at 48 hpf by dissolving the chorion envelope with secreted protease enzymes. During protease secretion, the chorion becomes thinner, eventually forming a hole that is enlarged by the developing fry's movement.

Blastula are more uniform in morphology compared to embryos at the gastrula stage when asynchronous development becomes evident. To examine penetration forces into lysed embryos, ten embryos were lysed by heating at the blastula stage in a petridish at a temperature of 40  $^{\circ}\text{C}$  for 20 min. The embryos were subsequently used in force measurements after being placed at room temperature (22  $^{\circ}\text{C}$ –24  $^{\circ}\text{C}$ ) for 2 h.

Besides quantitating mechanical property differences of zebrafish chorion at various developmental stages, another purpose of this study is to investigate the effect of pronase treatment on the chorion mechanical property modifications.

Embryos used for the pronase treatment experiments were treated with 2 units pronase/ml (Type XIV, Sigma Chem. Co) without shaking the embryos in zebrafish culture medium (ZCM) at the blastula stage at 3 hpf. At appropriate intervals, the treated embryos were washed with 10% FBS in ZCM three times to remove the remaining pronase for 5 min each. The chorion was completely dissolved by pronase treatment for 40 min [Fig. 2(d)]. Before force measurements were conducted, four embryo groups were incubated for 1, 3, 5, and 7 min in pronase-containing ZCM, respectively.

### B. Micromechanical Force Sensing System

The system consists of two micromanipulators (Fig. 3(e), model: MP-285, Sutter Inc.; Fig. 3(f), model: InjectMan NI2, Eppendorf) equipped with a holding pipette [Fig. 3(b)] and an injection pipette [Fig. 3(c)] on a vibration isolation table [Fig. 3(h)]. A precision  $x - y$  translation stage (Fig. 3(g), model: M-410DG, PI Inc., Germany) was used to produce planar motion with a large workspace under a stereomicroscope (Fig. 3(i), model: MZ-12.5, Leica Inc., Germany). The images of zebrafish embryo chorion deformations during pipette loading were captured in real time (30 Hz). A polyvinylidene fluoride (PVDF) piezoelectric force sensor [Fig. 3(d)] with a

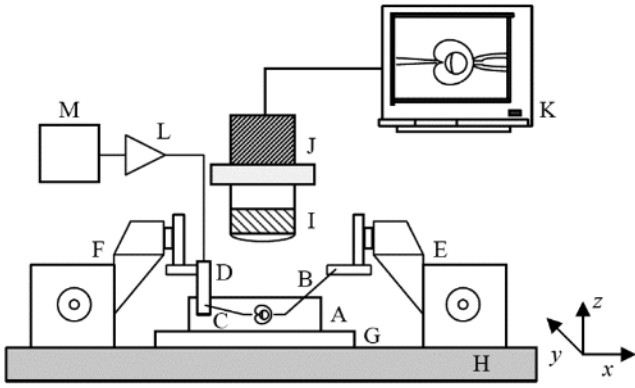


Fig. 3. A schematic of the micromechanical force sensing system for loading the chorion and measuring forces and chorion structural deformations. (a) Zebrafish embryo sample, (b) holding pipette, (c) injection pipette, (d) force transducer, (e and f) micromanipulator, (g) precision X-Y translation stage, (h) anti-vibration table, (i) microscope objective, (j) CCD camera, (k) image analysis system, (l) charge amplifier, and (m) data acquisition system are depicted on the scheme, respectively.

14.5- $\mu\text{N}$  resolution [22] was used to detect chorion penetration forces. The injection pipette was bonded to the tip of the PVDF force sensor that was clamped on a fixture on the micromanipulator [Fig. 3(f)]. Charges generated by the PVDF sensor were amplified by a charge amplifier [Fig. 3(l)]. The output was sampled through a data acquisition board (Fig. 3(m), model: dSPACE 1103), after which signals were digitized and filtered for noise rejection. The end tip of the injection pipette was 14.6  $\mu\text{m}$  in radius. The pipette motion was controlled at a speed of 120  $\mu\text{m/s}$ .

### C. Protein Gel Electrophoresis

To determine the chemical nature of “chorion softening” during prehatching stages, the chorions recovered from blastula embryos were partially digested for 10 min with 2 units pronase/ml to imitate the natural proteolytic activity caused by the embryo. The digested chorion solution along with nondigested chorion solution was collected and analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) [23] to compare the peptides before and after partial digestion. The gels were stained with 0.025% Coomassie Brilliant Blue.

## III. RESULTS AND DISCUSSION

Mechanical property changes of the chorion were found both during fertilization and at the prehatching stage. The first experiment was conducted on developing zebrafish embryos without pronase treatment. The chorion loading experiments were performed at room temperature (22–24  $^{\circ}\text{C}$ ). The force sensing profile and chorion structural deformations were obtained in real time during pipette penetration into the chorion envelope in blastula embryos. As shown in Fig. 4, the measured forces increased greatly during controlled chorion loading at a speed of 120  $\mu\text{m/s}$ . When deformation reaches approximately 170  $\mu\text{m}$ , the chorion envelopes at the blastula stage were punctured, resulting in the maximum penetration forces.

For embryos at the blastula stage, a mean penetration force of 737  $\mu\text{N}$  was measured, after which the force signal dropped to the basal level (Fig. 4).

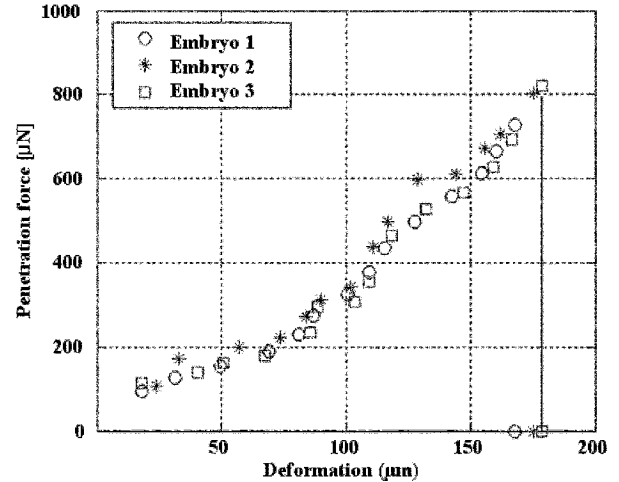


Fig. 4. Force-deformation curves of the zebrafish embryo chorion envelopes at the blastula stage. Experimental results of three embryos out of ten are shown. When deformation reached approximately 170  $\mu\text{m}$ , the chorion envelopes were punctured, which resulted in the maximum forces measured (penetration forces).

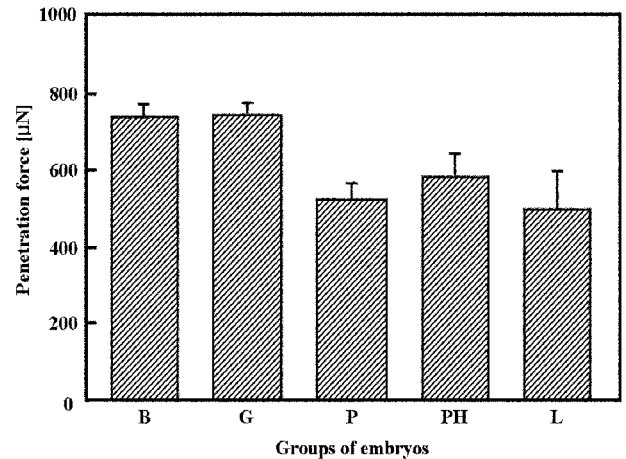


Fig. 5. Penetration force comparisons of the zebrafish embryo chorion at different developmental stages. Bars indicate values of measured average forces. Blastula (B), gastrula (G), pharyngula (P), prehatching (PH), and lyzed embryos (L) were used in each experiment. The data were obtained from the measurements in ten embryos in each group. To prepare the lyzed embryos with disorganized cytoplasm, blastula embryos were used because blastula embryos are more uniform in morphology compared to embryos at the gastrula stage when asynchronous development becomes evident such that the protease secretion effect of lyzed embryos can be more obviously revealed.

Force-deformation curves of embryos at other developmental stages produced similar patterns although measured penetration forces slightly varied (Fig. 5). The required average forces to penetrate chorion envelopes at the blastula stage (737  $\mu\text{N}$  with a standard deviation of 32.1  $\mu\text{N}$  ( $1\delta$ )) and gastrula stage (738  $\mu\text{N}$  with a standard deviation of 35.1  $\mu\text{N}$  ( $1\delta$ )) are 1.3-fold greater than those at the prehatching stage (578  $\mu\text{N}$  with a standard deviation of 63  $\mu\text{N}$  ( $1\delta$ )).

In order to estimate elastic modulus value changes of the zebrafish chorion at different developmental stages, a cell membrane mechanics model was used [6]. A deformed embryo membrane was approximated by a local dimple and a toroidal surface. Parameter values for the indentation of a zebrafish embryo

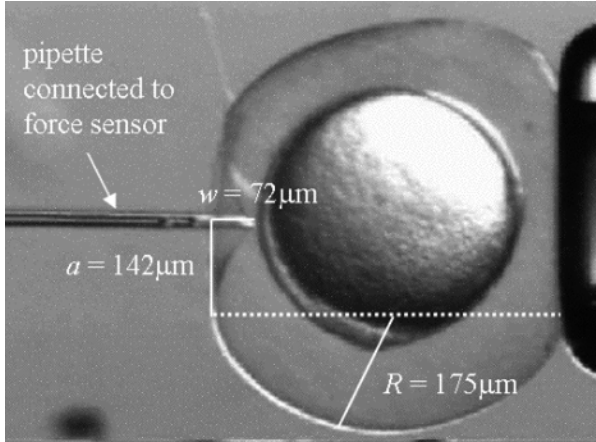


Fig. 6. Chorion loading and penetration. The pipette on the left is connected to the PVDF force sensor for force measurements. On the right is a holding pipette for fixing the cell. The geometrical parameters values are shown for the deformed chorion.

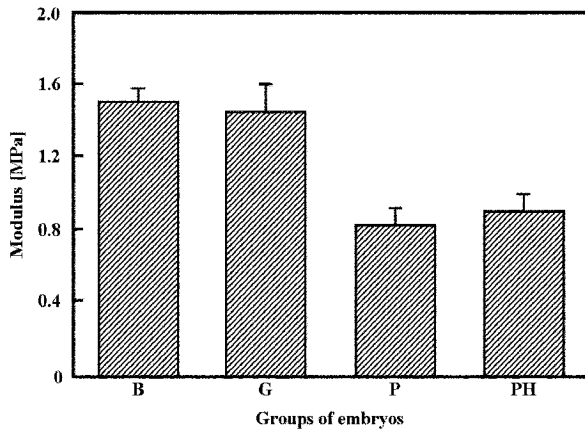


Fig. 7. Estimated elastic modulus values of the zebrafish embryo chorion at different developmental stages. Blastula (B), gastrula (G), pharyngula (P), and prehatching embryos (PH) were used in each experiment. The data were obtained from the measurements in ten embryos in each group.

by a pipette (cylindrical indenter) (Fig. 6) were determined in order to calculate elastic modulus values using

$$F = \frac{2\pi Ehw^3}{a^2(1-\nu)} \left[ \frac{3 - 4\zeta^2 + \zeta^4 + 2\ln \zeta^2}{(1-\zeta^2)(1-\zeta^2 + \ln \zeta^2)^3} \right] \quad (1)$$

where  $\zeta = c/a$ . The geometric parameters  $a$  and  $w$  were measured from captured images.  $F$  is the measured force. The poisson ratio  $\nu$  was assumed to be 0.5. The constants are  $3 \mu\text{m}$  for chorion's thickness ( $h$ ) and  $14.6 \mu\text{m}$  for indenter radius ( $c$ ). Elastic modulus ( $E$ ) based on 14 data points from embryos at the blastula and gastrula stages and nine data points from embryos at the pharyngula and prehatching stages were thus calculated. Throughout pipette loading, the modulus values were found to be fairly constant (Fig. 7). Elastic modulus values for individual chorion envelopes varied only slightly from the mean values. The mean elastic modulus value of the chorion envelopes at the blastula stage (1.51 MPa with a standard deviation of 0.07 MPa ( $1\delta$ )) was 1.66-fold greater than that at

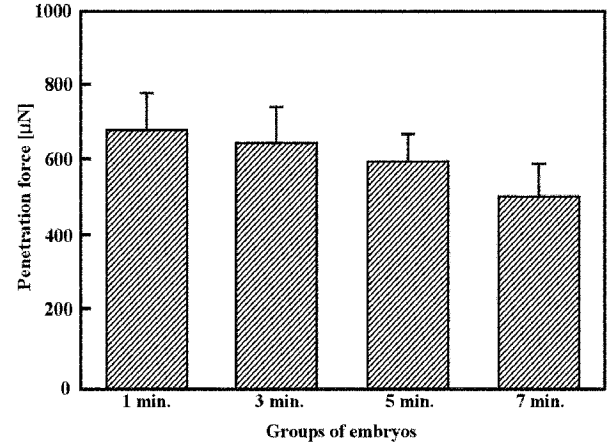


Fig. 8. Penetration force comparisons in the chorion envelopes of zebrafish embryos treated with pronase. Artificial "chorion softening" was achieved by pronase treatment. The embryos were treated with pronase for 1, 3, 5, and 7 min in the respective groups. The data were obtained from the measurements from ten embryos each.

the prehatching stage (0.91 MPa with a standard deviation of 0.09 MPa ( $1\delta$ )).

The second experiment was conducted to determine the mechanical properties of the lyzed zebrafish embryo chorion. Mean penetration forces of  $500 \mu\text{N}$  with a standard deviation of  $100 \mu\text{N}$  ( $1\delta$ ) were measured in ten lyzed embryos when the chorion envelopes were subjected to cell lysis of the embryos. It is known that lyzed embryos release cellular hydrolase including protease [14], [24]. Lyzed embryos are similar to the embryos at the prehatching stage in the sense that the chorion envelopes are both affected by protease activities by normal protease secretion (prehatching) and abrupt release of protease from dying cells (lyzed).

The third experiment was conducted to determine the effect of pronase treatment on mechanical properties of the zebrafish embryo chorion. Four groups of embryos were used in the experiment, each consisting of ten embryos. Each group was treated by pronase for 1, 3, 5, and 7 min, respectively. Note that the chorion envelopes of some embryos were swelling [arrows in Fig. 2(b) and Fig. 2(c)] or hatching [an arrow in Fig. 2(c) and all embryos in Fig. 2(d)] due to the action of the proteolytic enzyme, pronase. The chorion penetration forces of pronase-treated embryos revealed that lower forces were required for penetrating the pronase-treated chorion in a time-dependent manner (Fig. 8), which demonstrates that pronase treatment is capable of inducing an artificial "chorion softening" effect.

Finally, the electrophoretic analysis of the softened chorion by partial chorion digestion with pronase showed that fragments of ZP2 protein are present (Fig. 9), which was verified by a MALDI-TOF analysis of the protein fragments separated by gel electrophoresis (unpublished data). Therefore, it is predicted that as the hatching stage approaches, the proteolytic enzymes released from the embryo soften the chorion by degrading the major protein ZP2 into several peptide fragments. The other minor unknown proteins were not detected among the visible peptide bands in the separated gel of the partially digested chorion solution when stained with Coomassie blue. Therefore,

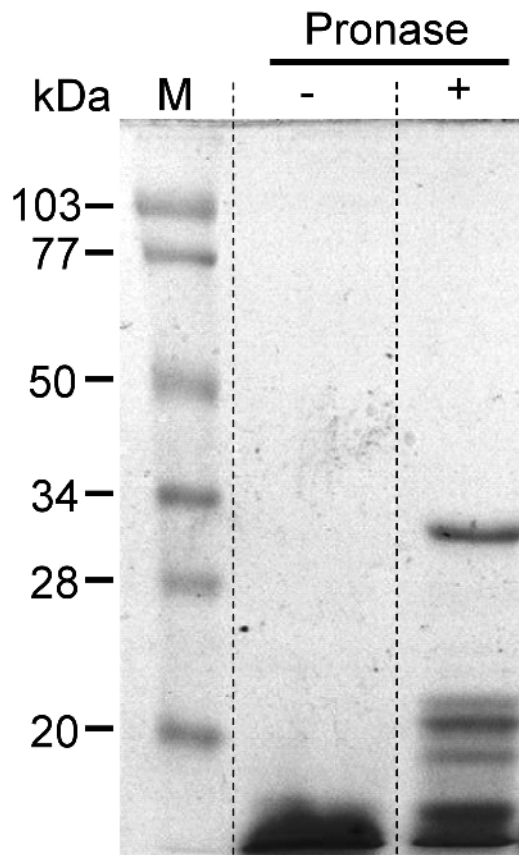


Fig. 9. Fragmentation of ZP2 protein in partially digested zebrafish chorion. To determine the chemical nature of “chorion softening” during prehatching stages, the chorions were treated with pronase for 10 min. The resulting chorion solutions without (–) or with pronase digestion (+) were separated by SDS-PAGE and stained with Coomassie Brilliant Blue to demonstrate peptide components present in the chorion solutions. M, molecular standard makers, and relative molecular masses (in kilo Daltons, kDa) are indicated on the left. A single protein band is seen in the lane without pronase, which is probably due to the presence of extremely abundant carbohydrates (anion charges) in the chorion. On the other hand, several peptide bands, apparently proteolytic products of the chorion, are present in the lane with pronase. The analysis of the peptide bands indicate that all major bands are derived from the ZP2 protein (see the text).

it appears that the spontaneously moving embryo at prehatching stage [Fig. 1(d)] should be able to make an opening in the weakened chorion having ZP2 fragmented during hatching process.

Thus, “chorion softening” was quantitated for both prehatching embryos and pronase-treated embryos, and its chemical nature was determined as a fragmentation of the ZP2 protein. In the prehatching embryos, the lower penetration forces are most likely due to proteolytic activity. Interestingly, the gradual decrease of penetration forces in pronase-treated embryo in a time-dependent manner suggests that pronase modification produces effects similar to the effects due to the gradual increase of proteolytic activity in prehatching embryos. These are the first experimental evidences that mechanical property modifications of the chorion are due to the proteolytic activities, and that ZP2 fragmentation occurs during the alteration. The results quantitatively demonstrate that mechanical property differences during “chorion softening” are most likely

due to proteolytic enzymes secreted by embryos. This chemical process involves the fragmentation of the ZP2 protein as confirmed by the protein gel electrophoresis results.

#### IV. CONCLUSION

The reported results in this paper present a quantitative study of zebrafish chorion mechanical modifications during early development. Through the use of a micromechanical force sensing system and a biomembrane elastic model, penetration forces and chorion elastic modulus values at various developmental stages were determined. Decreases in penetration forces and elastic modulus values were found during zebrafish embryo development. “Chorion softening” is most likely caused by proteolytic activities at the prehatching stage. This study also revealed the effect of pronase treatment on zebrafish embryo chorion, which produces an artificial “chorion softening” effect. An analogy was drawn between pronase treatment and proteolytic activities at the prehatching stage. Quantitating mechanical modifications of the zebrafish embryo chorion at different developmental stages provides a better understanding of zebrafish embryo development.

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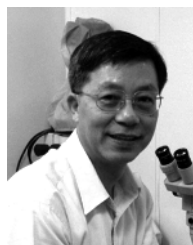
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