Two-dimensional gel electrophoresis of the organic matrix in chicken eggshell

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SUMMARY

Using 2D-PAGE and SDS-PAGE, we elucidated the structure of the organic matrix in chicken eggshell for the first time. The large variety of components contained implies that the eggshell formation may be controlled by several components. We believe that 2D-PAGE is a good tool for separating the heterogeneous organic matrix and can be used to analyze the organic matrix in different kinds of biominerals.

Key words: 2D-PAGE, organic matrix, chicken eggshell, biomineralization.

INTRODUCTION

Biominerals contain an organic matrix which is closely related to biomineralization. The organic matrix can usually be separated into two broad classes: the water-soluble matrix (WSM) and the water-insoluble matrix (WISM). However, few analyses have been carried out concerning the structure of the WSM in eggshells using column chromatography and amino acid analysis. Due to the complexity of the components in the WSM, their structures and functions cannot be successfully explained. On the other hand, little attention has been given to the WISM, primarily due to the difficulties associated with solubilization of it.

This study was undertaken to obtain basic information on the molecular weights and isoelectric points of the components in the WSM and WISM isolated from eggshell by SDS-PAGE and 2D-PAGE. This is the first attempt to use 2D-PAGE on the matrix protein in biominerals. Application of this method to the analysis of matrix proteins may serve as a good tool for understanding the mechanism of biomineralization.

MATERIALS AND METHODS

Material: Fresh chicken eggs were supplied from the Laboratory of Animal Resources and Breeding of the School of Veterinary Medicine, Azabu University. To remove cuticle, shells were washed thoroughly in running tap water and the inner membrane was peeled off from the shell. The remaining outer membrane was physically cleaned by cutter from the inner shell surface. Following these treatments, eggshell fragments were dipped in 10% EDTA (pH 7.8) for 5 min and then washed...

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Abbreviations: WSM, water-soluble organic matrix; WISM, water-insoluble organic matrix; C.B.B., coomassie brilliant blue.
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repeatedly with distilled water (DW) to remove the organic contaminant.

**Extraction:** Cleaned eggshell fragments (10 g) were dried, ground into powder by rotermill and decalcified in 10% EDTA under pH 7.8. After decalcification, salts were removed by dialysis against DW in Spectrapor No. 3 tubing (Spectrun Medical Ind., USA). The dialysate was centrifuged at 12,000 g and separated into the supernatant (WSM) and precipitate (WISM). Both components were freeze-dried and used for the analysis.

**SDS-PAGE:** Samples for SDS-PAGE were prepared in SDS sample buffer (1 mg WSM/100 µl sample buffer) and urea sample buffer (1 mg WISM/100 µl sample buffer). SDS-PAGE was carried out by the method of Laemmli, using ready made gel (10–20% gradient gel, Pagel/NPG-1020L, Atto Co., Japan). Gels were stained with silver stain (Silver stain II Kit Wako, Wako Co., Japan). Molecular weights of samples were compared with SDS-PAGE Standard Low (BIO-RAD Laboratories, Japan).

**2D-PAGE:** Samples for 2D-PAGE were prepared in urea sample buffer (about 1 mg samples/100 µl sample buffer). 2D-PAGE was carried out according to the method of O’Farrell. Gels for isoelectric focusing (ampholine, pH 3–10) were prepared in glass tubing (85×1.68 mm) and were run at 400 V for 90 min by an electrophoresis system for disc gel (Marysol, KS-8110, Marysol Co., Japan). Two-dimensional SDS-PAGE was carried out using ready-made gel for 2D-PAGE (10–20% gradient gel, Pagel/ NPG-1020D, Atto Co., Japan). The polyacrylamide gels were fixed with a solution of 13% trichloroacetic acid, 3.5% sulfosalicylic acid and 35% methanol and stained with silver stain. The pI range of each spot was compared with a carbamylete calibration kit for 2D-Electrophoresis (Pharmacia Biotech. Co., U. S. A.).

**Amino acid analysis:** Samples for the amino acid analysis were hydrolyzed in an ampoule under vacuum at 110°C for 24 h in 6N HCl. The hydrolysate was then analyzed on a Beckman Gold System-DABS26 automatic amino acid analyzer (Beckman Co., Japan).

**RESULTS**

SDS-PAGE under denaturating conditions (see Fig. 1) demonstrated that the WSM contained 13 bands detectable with silver stain at relative molecular weight of 75 kD to 10 kD. In contrast, the WISM contained 10 bands with molecular weight of 97 kD to 10 kD. The bands of 17 kD for the WSM and 97 kD, 45 kD and 17 kD for the WISM were strongly stained. While 8 bands were closely comparable between the two matrices, 2 bands with molecular weight of 75 kD and 66 kD were WSM specific and 2 bands with 97 kD and 56 kD were the WISM specific.

2D-PAGE patterns of the WSM and WISM (Fig. 2) resulted in 61 and 37 spots respectively, most of them located in the acidic pH area. The closely comparable spots between the two matrices were concentrated at the area of 38 kD with the pH between pH 4.8 to 6.0. The 17 kD component, which was well stained by SDS-PAGE of the two matrices, was also comparable in 2D-PAGE, shifting to the more basic pH area of pH 6.4 to 6.7. Moreover, spots with molecular weight of 97 kD with pI ranging between pH 6.0 to 6.8 were WISM specific, while elongated spots of 66 kD with pI ranging between pH 5.5 to 6.5 and basic spots with pI higher than pH 6.7 and 75 kD were WSM specific.

Amino acid compositions of the WSM and WISM (Table 1) were fundamentally similar. The most dominant amino acid was Gly and relatively high contents of Glx, Asx, Ala and Val were observed in both samples. In addition, the total contents of the...
acidic amino acids exceeded those of the basic amino acids. On the other hand, minor differences was found in the contents of several amino acids, such as higher values of Cys, Met, Tyr, Phe, Lys and Arg, and lower values of Thr and Ser in the WISM.

**DISCUSSION**

Until now little has been known about the structure and function of the organic matrix in eggshell. We tried to elucidate this problem by investigating separately the WSM and WISM. In contrast to the result of Somiya et al.6) who analyzed the eggshell matrix in rhea and demonstrated that the major component of the WSM was an unusually low molecular weight of less than 20 kD, we found the presence of many components with a wide range of molecular weights. The inconsistency may be the result of alteration because of the samples used by them were not fresh. The completely consistent result of SDS-PAGE following each rot of analysis indicates that this is the first record relating to the molecular weight of each component in the eggshell based on intact samples. The large variety of components contained implies that the eggshell formation may be controlled by the combination of several components in contrast to the case of mol-uluscan shell formation.7) As is generally agreed,
biominerals can be formed through several steps such as initiation, growth and termination stages, and it is easy to suppose that different matrix components play specific roles at each stage of eggshell formation. For example, a 97 kD component with pI of pH 6.0 to 6.8 may be the key component for the process of insolubilization of the matrix, which may be fully soluble just after secretion from the cells. Although the amino acid composition of the whole matrix was fundamentally similar between the WSM and WISM, the minor differences observed may be responsible for the solubility of the matrix. For example, Cys and Met, which were detected only from the WISM, may be important for constituting the secondary structure by forming S-S bonding. As the amino acid analysis of each matrix component has not yet been done, the cause of the minor differences remain unclear. Moreover, the higher ratio of the acidic to basic amino acids in the WSM is a subject of some interest as the acidic amino acids can bind Ca²⁺-ions and may be involved in the initial stage of eggshell formation. On the other hand, some of the components may be related to the contamination derived from the mother solution in eggshell gland. Recently, Miyamoto et al. clarified the structure of one component with molecular weight of 60 kD in the WSM in pearl. It was composed of two domains, one was CA-like and the other was an acidic domain. Based on immunological analysis, we found that specific components with molecular weights between 65 kD and 45 kD from several biominerals such as crocodile eggshell, fish otolith and molluscan shell showed reaction with the antiserum raised from the eggshell matrix (data not shown). These results imply the wide distribution of the key components for biomineralization. Thus, our next step of analysis must be focused on identifying the matrix protein related to the eggshell formation and on the characterization of the structure and function. We believe that 2D-PAGE is a good tool for separating the heterogeneous matrix component and can be applied to the organic matrix in a wide variety of biominerals.

REFERENCES
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