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Relationship between eggshell strength and keratan sulfate of eggshell membranes

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Abstract

Eggshell strength is an important factor in an effort to minimize eggshell breakage, which is a significant problem in the egg production industry. In the current study, we isolated and quantified the specific glycosaminoglycans (GAGs) from the calcified eggshell and shell membranes, which are related to eggshell strength. Our data suggest that GAGs exist in calcified eggshell may influence morphology of shell but do not affect on increase of shell amount while GAGs of shell membranes are maybe highly associated with shell strength with an increase of shell weight. Shell strength showed a strong correlation with the content of GAGs ($r=0.942$, $p<0.0005$) and a weak relationship with uronic acid content ($r=0.564$, $p=0.056$) in shell membranes. Monosaccharides in shell membranes were determined by Bio-LC® analysis for the identification of any specific GAGs related with shell strength. It indicates that the galactose content as a component of keratan sulfate (KS) has a significant correlation with eggshell strength ($r=0.985$, $p<0.0005$). These results suggest that eggshell strength is proportional to the KS content of eggshell membranes with an increase of eggshell weight.
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1. Introduction

Eggshell strength is an important factor to minimize the incidence of eggshell breakage, which is a big problem in egg producing industry. The eggshell is composed of a bilayered membrane and calcified extracellular matrix. It is clear that a linear relationship exists between the strength of shell and its mineral amount. However, this factor accounts for only a fraction of the shell resistance. In other calcified tissues, the organic components influence the organization and deposition of calcium salts and determine the crystal structure and strength of the material by modulating crystal nucleation and growth (Weiner and Addadi, 1991). The organic matrix may also exert some control on the biochemical properties of the eggshell by influencing the fabric or microstructure in the mineralization processes. To improve eggshell quality, therefore, it is necessary to characterize the molecular constituents involved in the mineralization of eggshell.

Mineralization is processed during the passages of eggs through the oviduct, with the layers of eggshell assembled sequentially as eggs pass through the arranged region of the oviduct (Arias et al., 1991a; Heuer et al., 1992). This formation is regulated by a precise spatio–temporal arrangement of sequentially deposited macromolecules (Fernandez et al., 1997). Morphologically, the eggshell is composed of shell membranes, mammillary knobs, palisades, and cuticles (Arias et al., 1992, 1993). They are shaped by combining particular extracellular matrix macromolecules with a crystalline calcite filler while the egg moves along the oviduct to produce a mineral–organic composite (Arias and Fernandez, 2001).

Previous reports show that various macromolecules in the calcified eggshell and shell membranes, which are composed of collagens, glycosaminoglycans (GAGs), proteoglycans (PGs) and diverse proteins, are involved in controlling eggshell mineralization (Arias et al., 1991b, 1992, 1997; Hincke et al., 1995, 1999, 2000, 2003; Carrino et al., 1996, 1997; Fernandez

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et al., 1997; Panheleux et al., 1999; Gautron et al., 2001a,b; Nakano et al., 2001, 2002; Nys et al., 2004). A quantitative relationship between levels of eggshell matrix proteins and eggshell quality are demonstrated in the previous reports (Panheleux et al., 2000; Ahmed et al., 2005), but a quantitative analysis of GAGs according to the eggshell quality has not been reported.

A comparative analysis of different biological mineralization models, such as shells, bones and teeth, indicate that GAGs and PGs play a role in mineralization (Blumenthal et al., 1979; Chen et al., 1984; Cuervo et al., 1973; Di Salvo and Schubert, 1967; Dziewiatkowski and Majznerski, 1985; Hunter et al., 1987; Weiner and Addadi, 1991). GAGs are acidic polysaccharides composed of repeating disaccharide units of amino sugar (D-glucosamine or D-galactosamine) and uronic acid (D-glucuronic acid or L-iduronic acid) or galactose (in keratan sulfate, KS). The shell matrices, which are the organic part obtained after a complete decalcification of eggshell, contain hyaluronic acid (Nakano et al., 2001) and dermatan sulfate proteoglycan (Arias et al., 1992; Carrino et al., 1996, 1997). The mammillary layer consists of mammillae containing mammillan, a highly sulfated keratan sulfate proteoglycan (Arias et al., 1992; Fernandez et al., 1997).

According to a previous report, eggshell strength depends on the GAGs content in an eggshell (Bronsch and Diamantstein, 1965). However, our previous studies on acidic polysaccharides in hard-shelled, soft-shelled and shell-less eggs showed that the concentration of acidic polysaccharides in soft-shelled eggs was higher than those in hard-shelled eggs (unpublished result). Specific GAGs responsible for the strength of eggshell have not yet been well established. Therefore, the present study focused on the relationship of GAGs, which are distributed in both calcified eggshell and membranes, and eggshell strength. We also investigated specific GAGs that are highly related to durability of the eggshell.

2. Materials and methods

2.1. Materials

Eggs were obtained from the College of Animal Husbandry of Konkuk University. Fifty five-week-old Hyline Brown laying hens (*Gallus gallus*) were used in the current study. Twenty hens were selected and housed individually in cages under normal condition. These birds were fed a standard diet containing 33 g calcium/kg. Acharan sulfate with a structure of a repeating unit disaccharide, α-L-iduronic acid-2-*O*-sulfate-α-D-*N*-acetylglucosamine was purified from the African giant *Achatina fulica* as previously described (Kim et al., 1996). Chondroitin sulfate, dermatan sulfate, (NZP Ltd., Palmerston North, New Zealand), and hyaluronic acid, D-glucuronolactone, D-galacturonic acid, D-galactosamine, D-glucosamine, *N*-acetylneuraminic acid, galactose and 1, 9-dimethyl-methylene blue (DMMB) (Sigma-Aldrich, St. Louis, MO, USA) were obtained for use as standards and reagents. Alcalase from *Bacillus subtilis* was a product obtained from NovoNordisk (Bagsvaerd, Denmark). Distilled water was filtered through a "NANO Pure Diamond" filtration system (Barnstead, IA, USA) before use. All other chemicals were of analytical grade.

2.2. Measurements of eggshell strength and weight

Egg weight was measured on all individual eggs before measurement of breaking strength. Breaking strength of uncracked eggs was measured with an FHK testing machine (Fujihara Co., Tokyo, Japan) and was recorded in maximum force (kgf/cm^2) required to crack the shell surface. The egg was placed on in its large end within a round indentation measuring 1 cm at the shallowest point and 2.5 cm at the deepest point. A pressure force was applied to the narrow end of the egg using a small anvil measuring 2.5 cm in width and 0.2 cm in thickness with a potential 50 kg load at a cross head speed of 50 mm/min (Hammerle, 1969). After testing, the egg content was discarded and the shells were washed, dried at room temperature. Then, eggshell membranes containing mammillary knobs were stripped from eggshell after immersion in 5% (w/v) EDTA for 1 h, followed by rinsing with distilled water and then dried in acetone. The dried calcified eggshell and shell membranes were weighed. Shell index (g/100 cm^2, Sauveur, 1988) was calculated as $I=(C/S)\times 100$, where C is a shell mass (calcified eggshell+eggshell membrane, g) and S is a shell surface (cm^2) with $S=4.68\times P^{2/3}$, when P = egg mass (g).

2.3. Isolation of glycosaminoglycans from calcified eggshell and membranes

Ninety six eggs were arranged in twelve groups according to the breaking strength to investigate correlations between the GAGs content and shell strength. The GAGs from the calcified eggshell and shell membranes containing mammillary knobs in each group were obtained for minimizing the effects of other molecules for the assay. The dried calcified eggshell and shell membranes were then homogenized separately using a Waring blender. The homogenized samples were decalcified by incubating with eight volumes of 25% glacial acetic acid at 4 °C for 24 h and dialyzed against distilled water. The decalcified samples were suspended in 0.05 M sodium carbonate buffer (pH 7.2). The suspensions were shaken for 48 h at 200 rpm at 60 °C after adding alcalase (*Bacillus subtilis*, 2.4 Anson units/g). The digestion mixtures were cooled to 4 °C and trichloroacetic acid was added to a final concentration of 5%. The samples were mixed, allowed to stand for 1 h, and then centrifuged for 20 min at 8000 ×*g*. The supernatants were recovered by decantation. Three volume of 5% potassium acetate in ethanol was added to one volume of supernatant. After mixing, the suspensions were stored overnight at 4 °C and then centrifuged for 30 min at 8000 ×*g*. The precipitates were then dissolved in 0.2 M NaCl and centrifuged for 30 min at 8000 ×*g*, and any insoluble materials were discarded. To the supernatant, cetylpyridinium chloride (5%) was added, and the precipitates were collected by centrifugation. The precipitates were dissolved in 2.5 M NaCl, 5 volume of ethanol were added and the samples were centrifuged for 30 min at 10,000 ×*g*. The precipitates were dissolved in water and dialyzed against 100 V

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