Chemical Composition of Chicken Eggshell and Shell Membranes

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ABSTRACT This study was undertaken to determine the occurrence of uronic acid in chicken eggshell membranes and to compare chemical compositions among the inner and outer eggshell membranes and the organic matter of eggshell. We report here for the first time the occurrence of uronic acid in chicken eggshell membranes. Uronic acid concentrations were similar ($P > 0.05$) between the inner shell membrane and outer shell membrane but approximately fivefold higher ($P < 0.05$) in the organic matter of eggshell. Sialic acid concentrations were the highest ($P < 0.05$) in the organic matter of eggshell and higher ($P < 0.05$) in the inner than in the outer shell membrane. Nitrogen concentrations were the lowest ($P < 0.05$) in the organic matter of eggshell but relatively constant between the two shell membranes. Amino acid analysis showed that the contents of glycine and alanine were higher ($P < 0.05$) and those of proline and hydroxyproline were lower ($P < 0.05$) in the organic matter of eggshell compared to shell membranes.

(*Key words*: uronic acid, sialic acid, eggshell, eggshell membrane, chicken)

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INTRODUCTION

The chicken eggshell comprises calcified shell and shell membranes including inner and outer membranes. These membranes retain albumen and prevent penetration of bacteria. Shell membranes are also essential for the formation of eggshell. The organic matter of eggshell and shell membranes contain proteins as major constituents with small amounts of carbohydrates and lipids (Burley and Vadehra, 1989). Bronsch and Diamantstein (1965) analyzed uronic acid in eggshells and reported a significant correlation between the uronic acid content and the breaking strength of the shell. Uronic acid is a constituent sugar of glycosaminoglycan. We have shown that the organic matter of eggshell contains two glycosaminoglycans including hyaluronic acid and chondroitin sulfate-dermatan sulfate copolymer. Sialic acid is another carbohydrate found in eggshell membranes (Itoh et al., 1990; Nakano et al., 1994). However, little is known about the difference in concentrations of sialic acid between the inner and outer eggshell membranes. There is also limited information available concerning variations in nitrogen concentrations and amino acid composition among the organic matter of eggshell and shell membranes. Eggshell and shell membranes are non-edible by-products with little saleable value. However, they may contain biologically active compounds. Better understanding of chemical composition of these by-products is of basic importance. Such knowledge may also be important for investigating the biological role of eggshell and shell membranes. This study was undertaken to analyze uronic acid, sialic acid, nitrogen, and amino acids in the organic matter of eggshell and the inner and outer shell membranes and to compare analytical data among these samples.

MATERIALS AND METHODS

Materials

Eggs were obtained from Single Comb White Leghorn hens reared under normal conditions at the University of Alberta Poultry Research Centre. Each egg was broken into half. Samples of inner and outer shell membranes were collected from the half shell containing the air cell. These membranes were separated from each other by applying air pressure through a small hole made on the inner membrane at the air cell. All samples of membrane were washed with deionized water and dried in acetone. The eggshells free of shell membrane were then decalcified by stirring with an excess of 0.5 *M* EDTA (tetrasodium salt), 0.05 *M* Tris, and 0.02% sodium azide, pH 7.2, at 4°C for 2 d. The mixture was then dialyzed in water and freeze-dried. Samples of decalcified eggshell or shell membranes were powdered using a blender and stored at 4°C until analyzed.

Determination of Uronic Acid

Samples of decalcified eggshell (approximately 0.1 g) were digested with papain as previously described (Na-

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kano et al., 2001). After proteolysis, cold trichloroacetic acid was added to a final concentration of 7% (wt/vol), to the digest, which was cooled to 4°C, and the mixture was left at 4°C overnight. Following removal of the precipitated protein by centrifugation, the supernatant was quantitatively transferred to a dialysis tube (molecular weight cutoff = 6,000 to 8,000), dialyzed against running tap water for 24 h, and then deionized water at 4°C for 24 h. Portions of the non-dialyzable fraction were used to determine uronic acid content by diphenyl reaction (Blumenkrantz and Asboe-Hansen, 1973) by using glucuronolactone as a standard.

Samples of inner and outer shell membranes (0.4 to 1.3 g), which were not appreciably digested with papain, were incubated at 55°C for 72 h with pronase-E[2] (1 g/g dry weight) in approximately 30 volumes of 0.05 *M* Tris-HCl, pH 8.0, containing 0.02% (wt/vol) sodium azide. After proteolysis, the digest was deproteinized with trichloroacetic acid as described above, and the trichloroacetic-acid-soluble fraction was dialyzed in water. Four volumes of ethanol containing 1% (wt/vol) sodium acetate was added to the dialysate. The mixture was left at 4°C overnight and centrifuged for 15 min at 20,000 × *g* and 4°C. The precipitate obtained was dried at 70°C for 10 min, dissolved in 1 mL of 0.5 *M* sodium acetate, pH 5.8, containing 0.02% (wt/vol) sodium azide (solution-A), and applied to a 1 × 114 cm gel chromatography column of Sephacryl S-300[3] equilibrated and eluted with solution-A. Fractions (~ 1 mL) collected at a flow rate of 9 mL/h were used to determine the content of uronic acid by diphenyl reaction (Blumenkrantz and Asboe-Hansen, 1973). The determination of uronic acid in each fraction was carried out on material that had been precipitated by addition of three volumes of 95% ethanol, stood overnight at 4°C, washed three times with 75% ethanol, and redissolved in a small volume of water. This procedure removed interfering substances such as sodium azide. The concentration of uronic acid in shell membrane was calculated based on the uronic acid recovered from the Sephacryl S-300 column. Gel chromatography was necessary for quantitative analysis of uronic acid because direct assay of uronic acid in the pronase digest resulted in formation of an anomalous (brown) color during the uronic acid assay with the diphenyl reaction. Materials that became brown were separated from uronic-acid-containing compounds on Sephacryl S-300 and eluted at or near the total column volume.

Other Methods

Determinations of moisture and ash contents followed the procedures described in AOAC (1998). Sialic acid was analyzed by the thiobarbituric acid reaction (Warren, 1959) after hydrolysis of samples in 0.1 *N* sulfuric acid at 80°C for 1 h. The chromophore formed was extracted using 1-propanol (Nakano et al., 1994) instead of cyclohexanone as used by Warren (1959). Nitrogen was analyzed by a LECO model F-428 nitrogen analyzer.[4] Analysis of amino acids with the exception of hydroxyproline was performed by using a Beckman Model 6300 amino acid analyzer[5] on samples hydrolyzed under nitrogen at 110°C for 24 h in glass-distilled 6 *N* HCl containing 0.1% (wt/vol) phenol. The hydroxyproline content was determined by the method of Stegemann and Stalder (1967). Digestions with *Streptomyces* hyaluronidase and chondroitinase-ABC were carried out as previously described (Nakano et al., 2001). Analysis of variance was used to detect significant ($P < 0.05$) differences between means of results, each of which was calculated based on three replicates.

RESULTS AND DISCUSSION

The dry decalcified eggshell and outer shell membrane contained 16.07 ± 1.43 and 0.31 ± 0.05% of ash, respectively, and the inner shell membrane contained an undetectable amount of ash. Analytical data expressed on an ash-free basis for the shell membranes and the decalcified eggshell are given in Table 1. The concentration of uronic acid was approximately fivefold higher ($P < 0.05$) in the decalcified eggshell than in the inner and outer shell membranes but was similar ($P > 0.05$) between the two membranes. Sialic acid concentrations were the highest ($P < 0.05$) in the decalcified eggshell and higher ($P < 0.05$) in the inner than in the outer shell membrane. The concentration of nitrogen was the lowest ($P < 0.05$) in the decalcified eggshell but similar ($P > 0.05$) between the two membranes.

Results of amino acid analysis are shown in Table 2. The contents of all amino acids (except for glycine, alanine, leucine, proline, and hydroxyproline) were similar ($P > 0.05$) among the decalcified eggshell and shell membranes. In contrast, the contents of glycine and alanine were higher ($P < 0.05$) and those of proline and hydroxyproline were lower ($P < 0.05$) in the decalcified eggshell compared to the shell membranes. The content of leucine was slightly higher ($P < 0.05$) in the decalcified eggshell than in the outer shell membrane but was similar ($P > 0.05$) between the two membranes.

Baker and Balch (1962) and Picard et al. (1973) reported no uronic acid present in the chicken eggshell. It is, however, unknown whether uronic acid was absent or present in a small amount that could not be detected in the previous studies. The present results show that chicken eggshell membranes contain relatively small but significant amounts of uronic acid. Baker and Balch (1962) extracted carbohydrates from chicken eggshell membrane using 1 *N* sulfuric acid at 100°C. This method without using proteolysis may not be appropriate to release sufficient amount of uronic acid from the shell membrane. Picard et al. (1973) treated eggshell membranes with pronase and trypsin and determined uronic acid content by a

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[3]Pharmacia Biotech Canada Inc., Baie d'Urfe, PQ, Canada.
[4]LECO Instruments Ltd., Mississauga ON, Canada.
[5]Beckman Coulter Canada Inc., Mississauga ON, Canada.

TABLE 1. Analysis of decalcified eggshell and eggshell membranes[1]

	Decalcified eggshell	Inner shell membrane	Outer shell membrane
Uronic acid	6.34 ± 0.20[a]	1.30 ± 0.10[b]	1.15 ± 0.18[b]
Sialic acid	4.83 ± 0.56[a]	1.70 ± 0.07[b]	0.48 ± 0.04[c]
Nitrogen	127.1 ± 6.9[a]	150.4 ± 2.4[b]	149.8 ± 1.4[b]

[a–c]Means in the same row with different superscripts are significantly ($P < 0.05$) different.
[1]Values are expressed as microgram per milligram of organic matter.

carbazole reaction in the fraction containing carbohydrate precipitated with cationic detergent cetylpyridinium chloride. It is not known if the fraction contained materials that interfered with the uronic acid assay. In the present study, Sephacryl S-300 chromatography was used to separate materials that interfere with the diphenyl reaction by producing anomalous chromophores. Another comment is that the carbazole method (Dische, 1947) used by Picard et al. (1973) may not be sensitive enough to detect uronic acid present. The method of Dische was later modified to improve its sensitivity by introducing sulfuric acid-sodium tetraborate solution (Bitter and Muir, 1962).

The uronic acid content found in the decalcified eggshell (Table 1) corresponded to approximately 0.03% of dry eggshell. This result is consistent with the concentration of uronic acid previously found in chicken eggshells (Nakano et al., 2001). The higher concentration of uronic acid observed in the decalcified eggshell than in the shell membrane suggests that anionic uronic acid may have an important role in mineralization of eggshell and, thus, in producing shells with high breaking strength as reported by Bronsch and Diamantstein (1965). The role of uronic acid in eggshell membranes is unknown.

Sephacryl S-300 chromatography (Figure 1) showed an average 36% of total recovered uronic acid eluted at or near the void volume of the column, and the remaining uronic acid eluted later, suggesting large variations of molecular sizes of the uronic-acid-containing compounds. In the decalcified eggshell, uronic-acid-containing compounds excluded from the column (fractions 35 to 43) were highly susceptible to *Streptomyces* hyaluronidase, an enzyme specific to hyaluronic acid (Ohya and Kaneko, 1970). Uronic-acid-containing compounds retarded in the column (fractions 44 to 70) were highly susceptible to chondroitinase-ABC, an enzyme that can degrade galactosaminoglycans including chondroitin sulfate and dermatan sulfate (Kresse and Glössl, 1987) (data not shown). Thus, the decalcified eggshell contained hyaluronic acid and galactosaminoglycans, confirming our previous report (Nakano et al., 2001). In contrast, uronic-acid-containing compounds from shell membranes were not appreciably susceptible to *Streptomyces* hyaluronidase or chondroitinase-ABC (data not shown). It remains to be investigated whether this result is due to the presence in the shell membranes of polysaccharides other than hyaluronic acid and galactosaminoglycan.

Itoh et al. (1990) reported that the sialic acid concentration in chicken eggshell is 2.7 μg/mg dry weight. This value appears to be much higher than the value (< 0.1 μg/mg) calculated from the present results (Table 1) assuming that the organic matter accounts for approximately 2% (Burley and Vadehra, 1989) of eggshell. The sialic acid concentration found in the outer membrane

TABLE 2. Amino acid composition of decalcified eggshell and eggshell membranes

Amino acid	Decalcified eggshell	Inner shell membrane	Outer shell membrane
	Mol%		
Asx	8.1 ± 0.1[a]	8.4 ± 0.4[a]	8.8 ± 0.1[a]
Thr	6.2 ± 0.1[a]	6.9 ± 0.0[a]	6.9 ± 0.2[a]
Ser	9.7 ± 0.1[a]	9.2 ± 0.2[a]	9.2 ± 0.0[a]
Glx	11.8 ± 0.2[a]	11.1 ± 0.4[a]	11.9 ± 0.3[a]
Gly	13.0 ± 0.3[a]	11.1 ± 0.2[b]	10.6 ± 0.2[b]
Ala	6.9 ± 0.4[a]	4.6 ± 0.2[b]	4.1 ± 0.2[b]
Val	7.3 ± 0.1[a]	7.2 ± 0.2[a]	7.9 ± 0.1[a]
Met	2.0 ± 0.2[a]	2.3 ± 1.0[a]	2.3 ± 1.0[a]
Ile	2.6 ± 0.1[a]	3.3 ± 0.4[a]	3.4 ± 0.1[a]
Leu	6.1 ± 0.2[a]	5.6 ± 0.5[a]	4.8 ± 0.2[b]
Tyr	1.8 ± 0.1[a]	2.2 ± 0.1[a]	1.7 ± 0.3[a]
Phe	2.1 ± 0.1[a]	1.6 ± 0.1[a]	1.5 ± 0.1[a]
His	4.2 ± 0.1[a]	4.1 ± 0.4[a]	4.3 ± 0.4[a]
Lys	3.6 ± 0.1[a]	3.6 ± 0.2[a]	3.4 ± 0.2[a]
Arg	5.9 ± 0.1[a]	5.7 ± 0.3[a]	5.8 ± 0.2[a]
Pro	8.3 ± 0.5[b]	11.6 ± 0.7[a]	12.0 ± 0.9[a]
Hyp[1]	0.3 ± 0.1[b]	1.5 ± 0.3[a]	1.4 ± 0.4[a]

[a,b]Means in the same row with different superscripts are significantly ($P < 0.05$) different.
[1]Hydroxyproline.

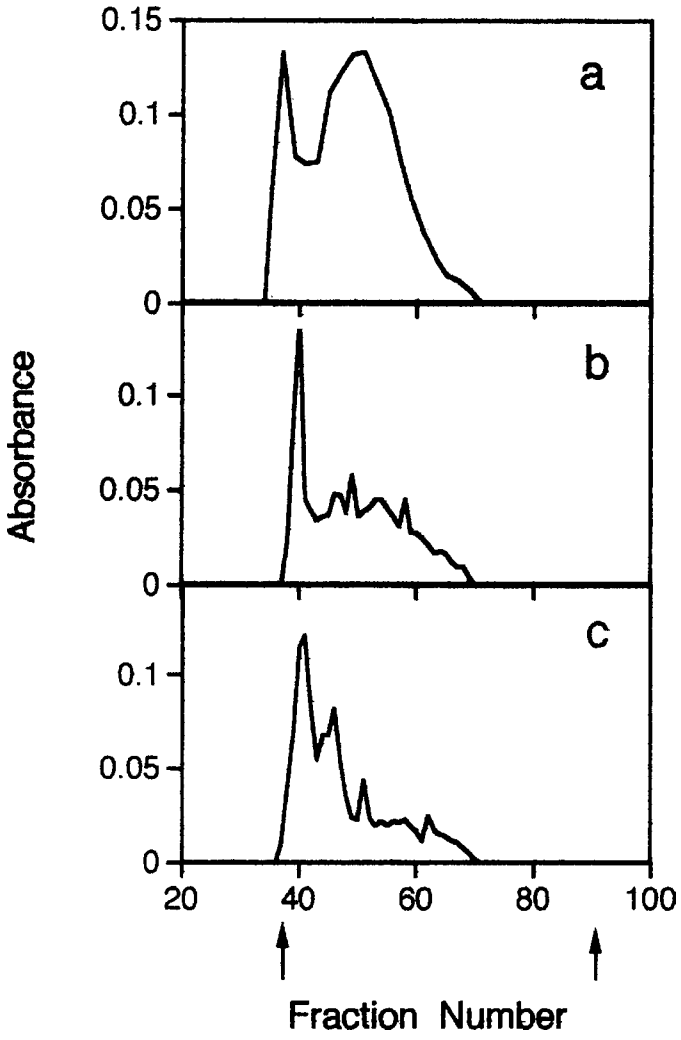

FIGURE 1. Sephacryl S-300 chromatography of proteinase digests of decalcified eggshell and eggshell membranes. Fractions collected were monitored for the content of uronic acid by the diphenyl reaction (absorbance at 520 nm). a) Papain digest of decalcified eggshell, b) pronase digest of inner shell membrane, and c) pronase digest of outer shell membrane. Positions of void volume and total column volume are shown by the first and second arrows, respectively.

(Table 1) is comparable to that (0.6 ± 0.1 μg/mg) found in samples containing both the inner and outer shell membranes, assuming that the dry weight of the inner membrane is much smaller (approximately sixfold; Cooke and Balch, 1970) than the outer membrane. The reason for the difference observed in sialic acid concentrations between the two membranes (Table 1) is not known. The nitrogen concentration found in the shell membranes (average 150.1 μg/mg, Table 1) was fairly comparable to that (155.4 μg/mg) reported by Baker and Balch (1962). The lower concentration of nitrogen in the organic matter of eggshell compared to shell membranes (Table 1) appears to reflect the lower concentrations of uronic acid and sialic acid in the latter.

Chicken eggshell membrane contains types I, V, and X collagens (Wong et al., 1984; Arias et al., 1992). In contrast, little is known about the occurrence of collagen in the organic matter of eggshell. The lower hydroxyproline content found in the decalcified eggshell than in shell membranes (Table 2) may suggest a higher collagen content in the latter. Contents of all amino acids, except hydroxyproline, determined in the decalcified eggshell and shell membranes were in general comparable with those reported in the literature (Candlish and Scougall, 1969; Salvesky and Leach, 1980).

At present, little is known about an efficient method for extraction of proteins and protein-polysaccharide complexes from eggshell membranes. Arias et al. (1992) extracted proteoglycans from eggshell membrane using 4 *M* guanidine hydrochloride and demonstrated the presence of keratan sulfate proteoglycan recognized by anti-keratan sulfate monoclonal antibody. However, these authors did not report the yield of the proteoglycan extracted. Development of a method for extracting uronic acid containing intact molecules from chicken eggshell membranes will facilitate the investigation of the structure of these unknown molecules.

In conclusion, the present study shows the occurrence of uronic acid in the chicken eggshell membranes. The results also demonstrate that chemical composition differs among the three structures with different functions including the fibrous eggshell membranes and the organic matter from mineralized eggshell.

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