Effect of different concentrations of calcium chloride and potassium chloride on egg white proteins during isoelectric precipitation of ovomucin

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ABSTRACT The effect of various concentrations of $CaCl_2$ and KCl on egg white proteins during isoelectric precipitation of ovomucin was investigated in this study. At low concentrations of $CaCl_2$ (<50 m*M*), lysozyme was the major contaminant in the precipitated ovomucin, whereas ovalbumin was the predominant one at high concentrations (≥100 m*M*). At 50 m*M* $CaCl_2$ concentration, the concentrations of both lysozyme and ovalbumin were moderate. Ovomucin with a purity of 97.3% was prepared using a 2-step method: egg white was first precipitated in the presence of 50 m*M* $CaCl_2$ followed by a second 500 m*M* $CaCl_2$ extraction. The concentrations of other proteins in the precipitate were 1.3% of ovalbumin, 1.1% of lysozyme, and 0.4% of ovomucoid. Unlike $CaCl_2$-treated samples, ovotransferrin was found to be the second major contaminant in all KCl-treated precipitates. Compared with the control, adding KCl at the lowest concentration of 2.5 m*M* increased significantly the content of ovalbumin (from 7.6 to 68.0%) and reduced significantly the content of lysozyme (from 25.5 to 6.4%) in the precipitates; however, increasing the concentrations of KCl up to 500 m*M* did not affect the content of ovalbumin, but the content of lysozyme showed a general reduction trend. Although KCl was used widely in literature as the last step of ovomucin washing, our results show that KCl is not an efficient salt in purifying ovomucin.

Key words: egg white, ovomucin, isoelectric precipitation, calcium chloride, potassium chloride

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INTRODUCTION

Ovomucin is a viscous glycoprotein that is responsible for the gel structure of thick egg white (Brooks and Hale, 1959). It accounts for 2 to 4% of egg white proteins (Donovan et al., 1970; Robinson and Monsey, 1971), consisting of a carbohydrate-poor subunit (α-ovomucin) and a carbohydrate-rich subunit (β-ovomucin) (Kato and Sato, 1971; Robinson and Monsey, 1971; Itoh et al., 1987). In addition to its excellent foaming and emulsifying properties (Kato et al., 1985), ovomucin was found to be the key factor for the inhibitory activity of egg white against hemagglutination by influenza viruses (Gottschalk and Lind, 1949); it was also reported to have antiviral and antitumor properties (Ohami et al., 1993; Tsuge et al., 1996, 1997; Watanabe et al., 1998). Although numerous attempts have been made to clarify its chemical and functional characteristics, ovomucin is one of the least defined egg proteins due to its difficulty in purification, poor solubility after isolation, and large molecular weight.

The most common method (Brooks and Hale, 1959) used for preparing ovomucin involves precipitation of diluted egg white followed by washing the precipitate first with water and then with 2% KCl. It has been reported by many researchers (Cotterill and Winter, 1955; Robinson and Monsey, 1972) that ovomucin could covalently interact with lysozyme. Hawthorne (1950) showed that ovomucin and lysozyme form an insoluble protein-protein complex and suggested that this complex plays an important role in egg white thinning. In the previous method of ovomucin preparation, a high concentration of KCl was used due to the fact that high salt concentration minimizes complex formation between ovomucin and other egg white proteins like lysozyme (Brooks and Hale, 1961). However, the KCl washing step was reported to cause loss of ovomucin (Robinson and Monsey, 1969). In literature, the purity of the prepared ovomucin by precipitation was found to be less than 70% due to the co-precipitation of other egg white proteins such as ovalbumin and lysozyme. For research purposes, pure ovomucin can be prepared by gel filtration method (Young and Gardner, 1972); however, this method is not suitable for large-scale pro-

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duction. In our previous report, we developed a new 2-step method to prepare ovomucin using NaCl solution (Omana and Wu, 2009): ovomucin was initially precipitated using 100 m*M* NaCl, followed by a second step of extraction using 500 m*M* NaCl. The resultant ovomucin has a purity of 94.6%, with slight contamination of ovalbumin (3.7%), ovomucoid (1.1%), and lysozyme (1.8%); the yield of ovomucin was 400.2 mg/100 g of egg albumen (Omana and Wu, 2009).

Our previous study showed that the protein compositions of ovomucin precipitates are significantly affected by the NaCl concentrations (Omana and Wu, 2009); however, the effects of $CaCl_2$ and KCl on the extractability of ovomucin have not been reported. Moreover, KCl was employed previously in an ovomucin washing step. Hence, the objective of the present study was to determine the effect of different concentrations of $CaCl_2$ and KCl on egg white proteins during the isoelectric precipitation of ovomucin.

MATERIALS AND METHODS

Materials

Fresh eggs of Lohmann White Single Comb White Leghorns (52 wk of age) were obtained from the farm of the Poultry Research Centre of University of Alberta in the morning and used for experiment within the same day. Standard proteins (ovalbumin, ovotransferrin, ovomucoid, and lysozyme) were obtained from Neova Technologies Inc. (Abbotsford, British Columbia, Canada). Sodium dihydrogen phosphate monohydrate, disodium orthophosphate heptahydrate, and HCl were purchased from Fisher Scientific (Ottawa, Ontario, Canada). 2-Mercaptoethanol and KCl were obtained from MP Biomedicals LLC (Solon, OH). Calcium chloride was purchased from Acros Organics (Morris Plains, NJ).

Effects of Salt Concentration on the Extraction of Ovomucin

Ovomucin was prepared as a function of $CaCl_2$ or KCl at concentrations of 0, 2.5, 5, 10, 50, 100, 200, and 500 m*M*. The treatment with 0 m*M* salt concentration was considered as control. The salt solutions were prepared in distilled water. All the preparations were made in duplicate. Egg white was manually separated from egg yolk and then homogenized using a stirrer for 30 min. To 180 mL of salt solution at various concentrations, 60 mL of homogenized egg white solution was added in 250-mL centrifuge tubes. The pH of the solution was then adjusted to 6.0 using 1 *N* or 0.1 *N* HCl, while stirring for 30 min. The centrifuge tubes were kept at 4°C overnight, and the precipitates (crude ovomucin) were then separated by centrifugation at 15,300 × *g* for 10 min at 4°C, using an Avanti J-E refrigerated centrifuge (Beckman Coulter Inc., Fullerton, CA), freeze-dried (Virtis freeze drier, The Virtis Company, Gardiner, NY), and stored at −20°C until analysis.

Preparation of Ovomucin by Precipitation with 50 mM $CaCl_2$ Followed by 500 mM $CaCl_2$ Solutions

Based on the results of the effects of different salt concentrations on the precipitation of various egg white proteins, it was found that 50 and 500 m*M* of $CaCl_2$ have a profound influence on the major co-precipitating proteins. Hence, a new protocol was developed for the preparation of ovomucin to enhance its purity (Figure 1). Ovomucin was first prepared using isoelectric precipitation in the presence of 50 m*M* $CaCl_2$ solution. The dispersion was kept overnight at 4°C and separated by centrifugation at 15,300 × *g* for 10 min at 4°C. The precipitate was further suspended in 500 m*M* $CaCl_2$ solution while stirring for 4 h followed by overnight settling at 4°C. After centrifugation at 15,300 × *g* for 10 min at 4°C, the precipitate was freeze-dried and stored at −20°C until analysis.

Preparation of Ovomucin by Conventional Method

Ovomucin was also prepared by the conventional method using the protocol given in Figure 2. Homogenized egg white was diluted with water and precipitated at pH 6.0. After overnight incubation at 4°C, the precipitate was separated by centrifugation at 15,300 × *g* for 10 min. The precipitate was then repeatedly washed with 2% KCl solution, until the supernatant became protein-free. Finally, the precipitate was separated again by centrifugation, freeze-dried, and stored at −20°C until use.

Yield Determination

Yield of ovomucin was calculated by taking into account the protein concentration and purity of ovomucin using the formula as follows:

$$\text{Yield} = \frac{W_1 \times \text{protein } \% \times \text{purity } \%}{100 \text{ g of egg white}}, \quad [1]$$

where W_1 = weight of freeze-dried ovomucin powder; protein % = protein content of freeze-dried ovomucin in percentage; and purity % = ovomucin purity obtained from gel filtration data in percentage. Percentage yield was also calculated as a ratio of ovomucin weight between ovomucin obtained and ovomucin in raw egg white by using the following formula:

$$\text{Yield} = \frac{W_1 \times \text{protein } \% \text{ (ovomucin powder)} \times \text{purity } \% \text{ (ovomucin powder)}}{W_2 \times \text{protein } \% \text{ (egg white)} \times \text{ovomucin purity } \% \text{ (egg white)}} \times 100, \quad [2]$$

where W_1 = weight of ovomucin powder prepared from W_2 of egg white and W_2 = weight of egg white required to prepare W_1.

Gel Filtration Chromatography

The purity of the prepared ovomucin was determined using a high-load 16/60 column (Superdex 200 preparative grade, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) coupled with fast-performance liquid chromatography equipment (AKTAexplorer, GE Healthcare Bio-Sciences AB) using the method as described by Hiidenhovi et al. (2002) with slight modifications. Instead of using 2 Superose 6 columns in series in the original method, we used a Superdex 200 GFC matrix. Samples, at a concentration of 5 mg/mL, were prepared by dissolving ovomucin in 100 m*M* sodium phosphate buffer (pH 7.0) containing SDS (50 mg/mL) and β-mercaptoethanol (10 μL/mL) by overnight stirring (200 rpm) at ambient (22°C) temperature using an IKA RCT B S1 stirrer (IKA Works Inc., Wilmington, NC). The samples were filtered through a cellulose syringe filter (0.45 μm, Fisher Scientific). The injection volume was 3 mL and the column was eluted with 100 m*M* of phosphate buffer (pH 7.0) containing SDS (5 mg/mL) and β-mercaptoethanol (1 μL/mL) at a flow rate of 1 mL/min monitoring at 280 nm.

The concentration of co-precipitated proteins was calculated from standard protein curves and the concentration of ovomucin was calculated by subtracting the amount of co-precipitated proteins according to Hiidenhovi et al. (2002). All the determinations were carried out in duplicate.

SDS-PAGE

Sodium dodecyl sulfate-PAGE was carried out according to Laemmli (1970) using a Bio-Rad electrophoresis system consisting of 10 to 20% ready gels at constant voltage mode in a Mini-PROTEAN tetra cell attached to a PowerPac Basic electrophoresis apparatus (Bio-Rad Laboratories Inc., Hercules, CA). The loaded amount of proteins was 50 μg for all the samples. Protein markers of high range molecular weight obtained from Bio-Rad were loaded into a separate well for comparison of molecular weight. The gels were scanned using an Alpha Innotech gel scanner with FluorChem SP software (Alpha Innotech Corp., San Leandro, CA).

Protein Content

Protein content of freeze-dried ovomucin powders and egg white was determined for yield calculation. Protein content of all the samples was determined using a TruSpec CN carbon-nitrogen determinator (Leco Corp., St. Joseph, MI) and multiplying nitrogen content by a factor of 6.25. Analyses were carried out in duplicate.

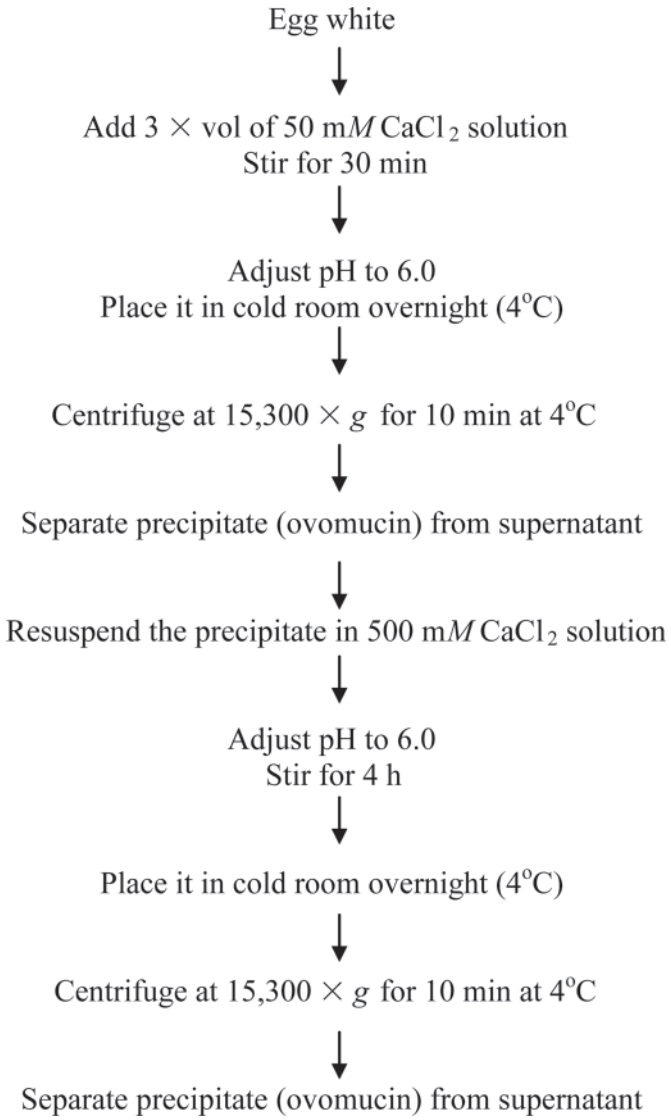

Figure 1. Protocol of ovomucin preparation by precipitation using 50 m*M* $CaCl_2$ followed by 500 m*M* $CaCl_2$ solution.

Statistical Analysis

Different treatments were subjected to ANOVA using the GLM procedure of SAS (SAS version 9.0, SAS Institute, Cary, NC). Means were separated using Duncan's multiple range test at a level of significance of $P < 0.05$ (Duncan, 1955).

RESULTS AND DISCUSSION

Effect of Different Concentrations of $CaCl_2$ on Protein Composition of Ovomucin

Isoelectric precipitation is commonly employed in the preparation of ovomucin (Brooks and Hale, 1961; Kato et al., 1970) based on its minimum solubility at this pH. It is well-known that salts affect the electrostatic interactions among the proteins and protein solvent (Damodaran, 1996); therefore, solubility of different proteins in salt solutions is a function of the ionic spe-

cies and concentration (Arakawa and Timasheff, 1982). The protein composition of the ovomucin precipitates prepared using isoelectric precipitation at different concentrations of $CaCl_2$ was analyzed by gel filtration chromatography (Figure 3). In the conventional method of ovomucin preparation, the major contaminating proteins were ovalbumin and lysozyme. As evident from gel filtration chromatogram, ovalbumin was increasing at increasing $CaCl_2$ concentrations, whereas lysozyme showed the opposite trend.

Protein composition of ovomucin precipitates prepared at different concentrations of $CaCl_2$ is given in Table 1. In general, the lysozyme concentration in the precipitates was decreased, whereas ovalbumin was increased at increasing salt concentrations. Compared with the control (0 m*M* $CaCl_2$), the lysozyme concentration in the precipitate was slightly higher at low salt concentrations but was dramatically reduced 5 times at concentrations greater than 50 m*M*; on the contrary, the concentration of ovalbumin was increased to over 5 times at concentrations greater than 50 m*M*. Ovomucoid was found to have marginal decrease with increasing salt concentrations but was below detection level at salt concentrations $\geq$100 m*M*. The purity of the precipitated ovomucin might be slightly overestimated in the 0 to 100 m*M* $CaCl_2$-treated samples due to the overlap of ovotransferrin with ovomucin. Ovotransferrin was found to be measurable only at 200 and 500 m*M* salt concentrations. These results have the same trend as our previous study with different concentrations of NaCl (Omana and Wu, 2009). But in the case of NaCl-treated samples, 100 m*M* NaCl was found to be critical; above this concentration, there was a drastic increase in ovalbumin and decrease in lysozyme content. Effect of different $CaCl_2$ concentrations on the protein composition of ovomucin extracts were further analyzed by SDS-PAGE (Figure 4). Our results closely related to the ovomucin reported by Itoh et al. (1987), in which the molecular weight of β, $\alpha 2$, and $\alpha 1$ were found to be 400,000, 220,000, and 150,000 Da, respectively. The trend that the concentration of ovalbumin was increased whereas that of lysozyme was decreased in the ovomucin extracts at increasing $CaCl_2$ concentrations up to 500 m*M* was evident from the intensity of the corresponding bands, which is consistent with the results of gel filtration analysis.

Yield of ovomucin prepared as a function of $CaCl_2$ concentrations varied from 107.9 to 267.8 mg/100 g of egg albumen, which corresponds to 24.9 to 62.1% of the total ovomucin present in the egg white (Table 2). The yield of control sample was found to be 260.4 mg/100 g of egg albumen, and the maximum yield (267.8 mg/100 g of egg albumen) was observed at a salt concentration of 2.5 m*M* $CaCl_2$ concentration. A wide range of ovomucin yield was reported in literature; comparison of the yields and compositions of isolated ovomucins is difficult because of variations in the methods of preparation (Osuga and Feeney, 1968; Donovan et al., 1970). The reported yield of ovomucin varied from 90 to 117 mg of ovomucin per 100 g of albumin by Brooks and

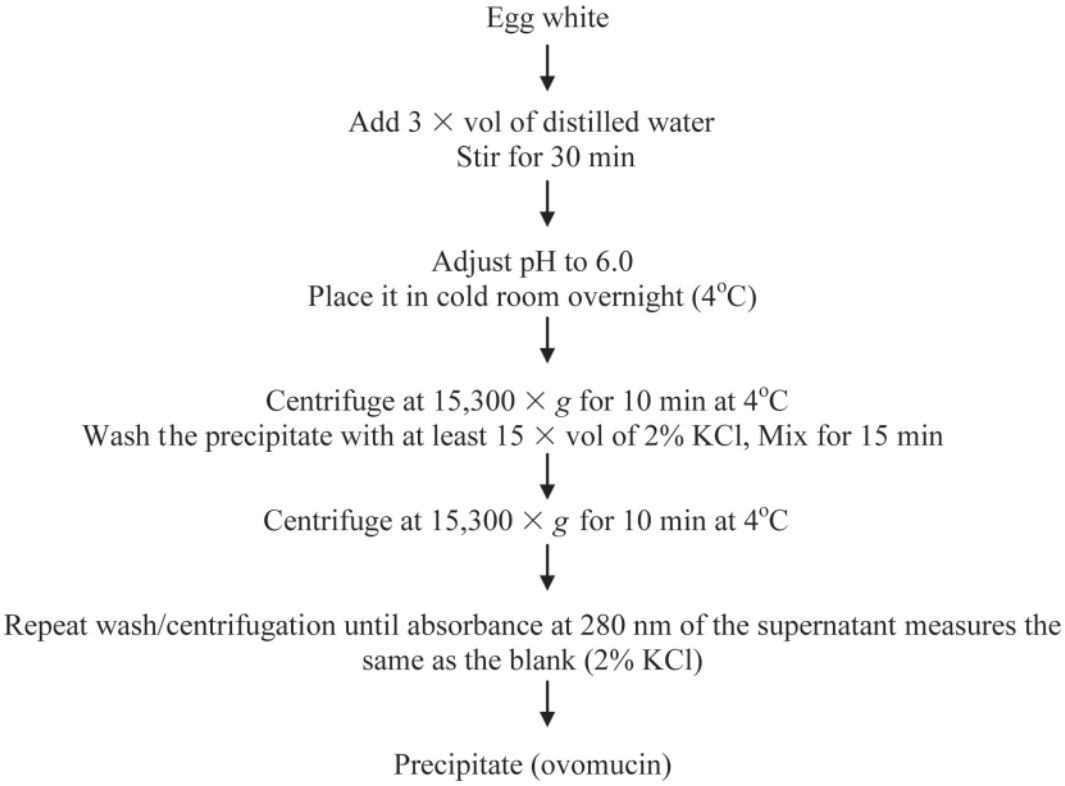

Figure 2. Protocol of ovomucin preparation by the conventional method.

Hale (1961) after an extensive washing procedure to 530 mg in 100 g of whole egg white (Toussant and Latshaw, 1999). In our earlier study of isoelectric precipitation of ovomucin with different concentrations of NaCl, yield varied from 217.7 to 330.0 mg/100 g of egg white (Omana and Wu, 2009).

Effect of Different Concentrations of KCl on Protein Composition of Ovomucin

The protein composition of the ovomucin precipitates prepared using isoelectric precipitation at different concentrations of KCl was analyzed by gel filtration chro-

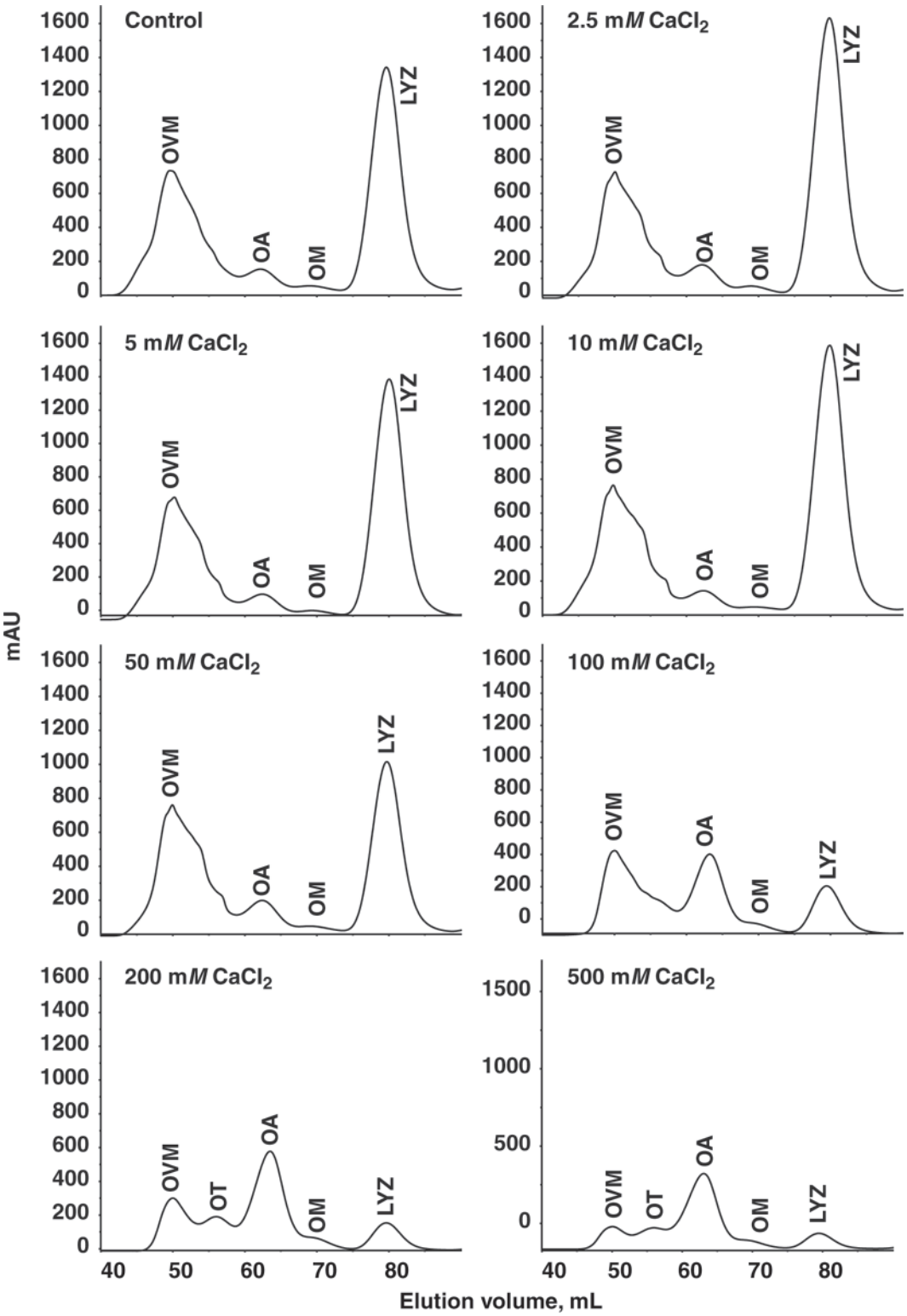

Figure 3. Gel filtration chromatogram of ovomucin prepared as a function of $CaCl_2$ concentrations. OVM = ovomucin; OT = ovotransferrin; OA = ovalbumin; OM = ovomucoid; LYZ = lysozyme.

Table 1. Protein composition of ovomucin samples prepared as a function of $CaCl_2$ concentrations[1]

Treatment	Ovotransferrin, %	Ovalbumin, %	Ovomucoid, %	Lysozyme, %	Ovomucin, %
Control, 0 m*M*	ND[2]	7.6^{de} (0.4)	3.2^{a} (0.8)	25.5^{d} (0.5)	63.8^{ab} (0.2)
2.5 m*M*	ND	11.3^{c} (1.3)	4.3^{a} (2.1)	30.5^{a} (0.4)	54.0^{d} (3.0)
5 m*M*	ND	8.3^{d} (1.4)	4.3^{a} (3.3)	27.2^{c} (1.0)	60.2^{bc} (3.8)
10 m*M*	ND	6.3^{e} (0.0)	1.5^{a} (0.5)	28.7^{b} (0.4)	63.5^{ab} (0.0)
50 m*M*	ND	11.5^{c} (0.5)	1.8^{a} (0.1)	19.0^{e} (0.0)	67.8^{a} (0.5)
100 m*M*	ND	38.8^{b} (0.5)	ND	5.2^{f} (0.0)	56.0^{cd} (0.4)
200 m*M*	8.8 (1.6)	46.2^{a} (0.0)	ND	3.0^{g} (0.5)	42.1^{f} (1.1)
500 m*M*	5.8 (0.8)	45.2^{a} (0.0)	ND	2.6^{g} (1.0)	46.5^{e} (1.8)

$^{a–g}$Means with different superscripts in the same column are significantly different ($P < 0.05$).

[1]Given values are mean values of duplicate trials, n = 2. Values in parentheses represent SD. Ovotransferrin could not be estimated in 0- to 100-m*M* samples due to overlap of peaks.

[2]ND = below detection level.

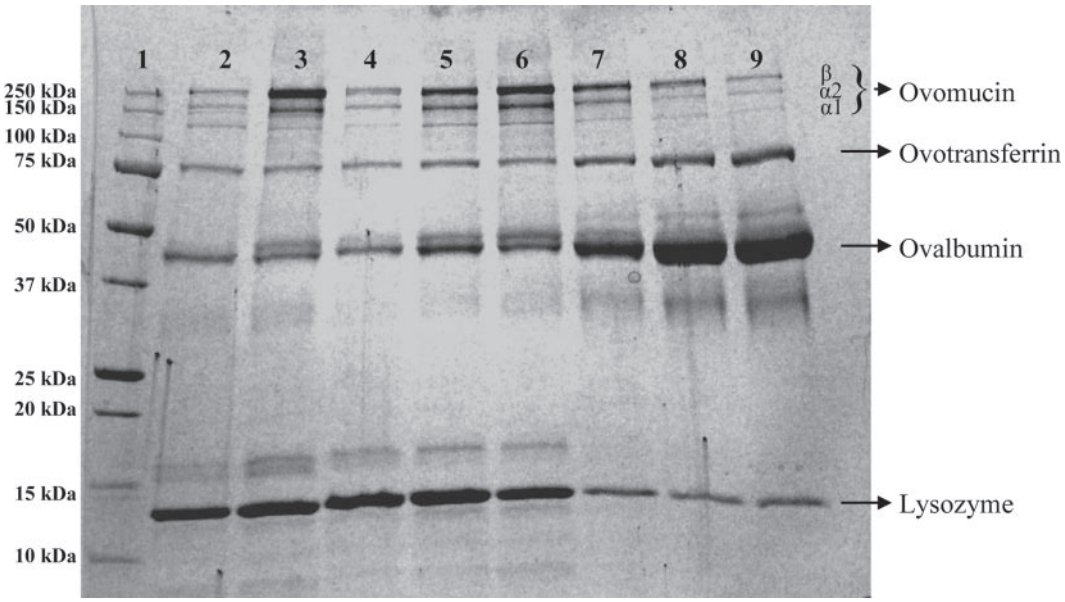

Figure 4. The SDS-PAGE pattern of proteins during ovomucin extraction using different $CaCl_2$ concentrations under reduced conditions. Note that an equal concentration of samples was loaded. Lane 1 = standard molecular weight marker; lane 2 = control, 0 m*M*; lane 3 = 2.5 m*M*; lane 4 = 5 m*M*; lane 5 = 10 m*M*; lane 6 = 50 m*M*; lane 7 = 100 m*M*; lane 8 = 200 m*M*; lane 9 = 500 m*M*.

Table 2. Yield of ovomucin prepared in the presence of $CaCl_2$[1]

Treatment	Ovomucin content, mg/100 g of egg albumen	Ovomucin expressed as percentage of ovomucin in egg white, %
Control, 0 m*M*	260.4^{a} (1.2)	64.8^{a} (0.2)
2.5 m*M*	267.8^{a} (21.3)	62.1^{a} (2.1)
5 m*M*	251.7^{a} (22.6)	58.5^{a} (2.2)
10 m*M*	169.2^{b} (0.0)	38.9^{b} (0.0)
50 m*M*	147.9^{bc}(1.5)	34.1^{bc} (0.2)
100 m*M*	107.9^{d} (1.1)	24.9^{d} (0.1)
200 m*M*	128.1^{cd}(4.9)	29.6^{cd} (0.5)
500 m*M*	135.9^{bcd} (7.6)	32.3^{bcd} (0.1)

$^{a–d}$Means with different superscripts in the same column are significantly different ($P < 0.05$).

[1]Given values are mean values of duplicate trials, n = 2. Values in parentheses represent SD.

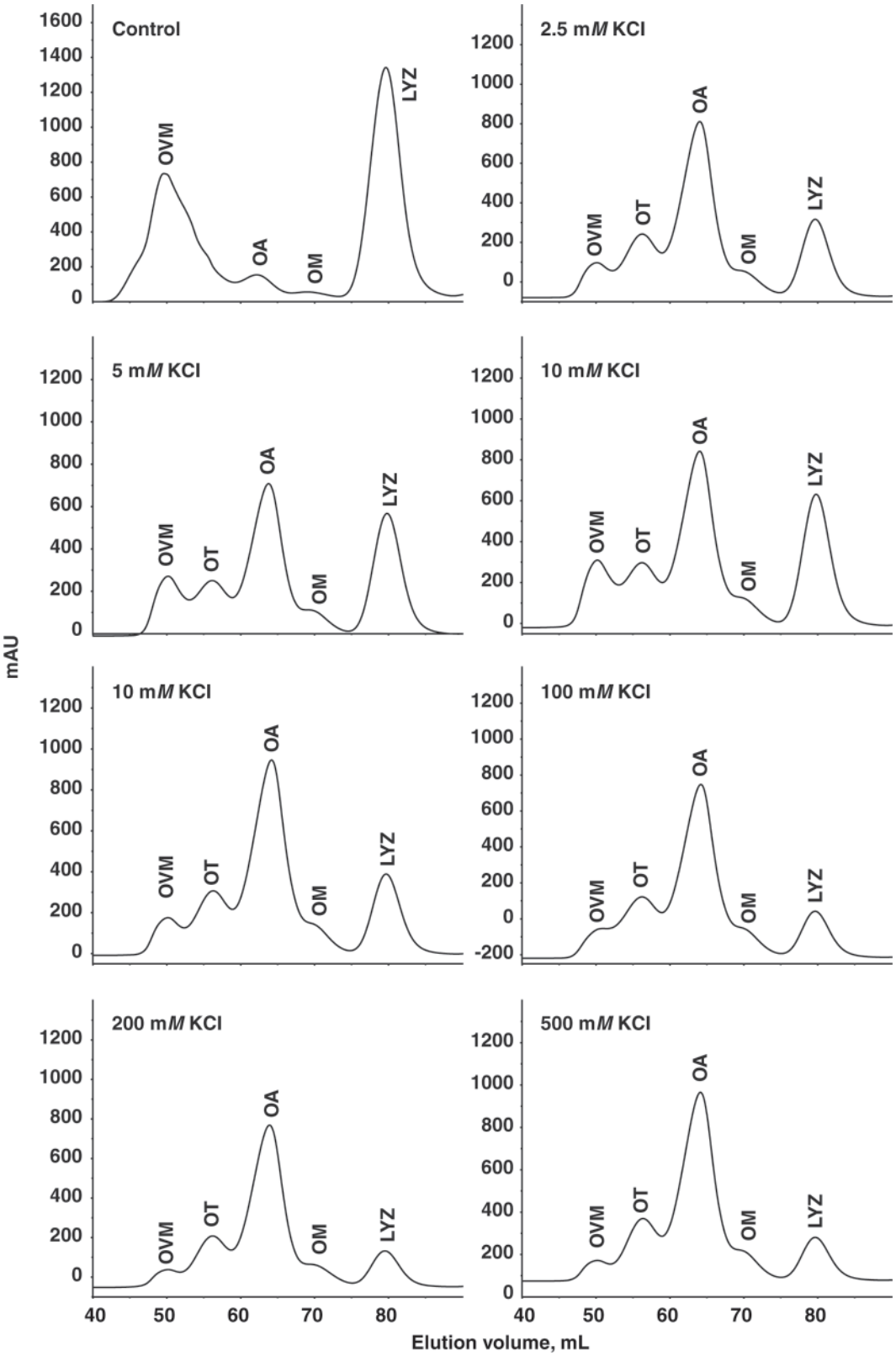

Figure 5. Gel filtration chromatogram of ovomucin prepared as a function of KCl concentrations. OVM = ovomucin; OT = ovotransferrin; OA = ovalbumin; OM = ovomucoid; LYZ = lysozyme.

matography (Figure 5). Our results showed that ovalbumin was the predominant protein in all KCl-treated samples, which is different from the trends observed with the addition of NaCl or $CaCl_2$. It has already been established that KCl is an effective salting-out agent for ovalbumin (Dumetz et al., 2007). Gel filtration analysis also showed the presence of ovotransferrin even at the low concentrations of KCl. Compared with the control,

Table 3. Protein composition of ovomucin samples prepared as a function of KCl concentrations[1]

Treatment	Ovotransferrin, %	Ovalbumin, %	Ovomucoid, %	Lysozyme, %	Ovomucin, %
Control, 0 m*M*	ND[2]	7.6[c] (0.4)	3.2 (0.8)	25.5[a] (0.5)	63.8[a] (0.2)
2.5 m*M*	12.3[a] (0.5)	68.0[a] (0.0)	ND	6.4[c] (0.1)	13.3[c] (0.4)
5 m*M*	9.0[a] (2.4)	50.7[b] (7.5)	ND	9.7[b] (0.5)	30.7[b] (10.4)
10 m*M*	10.8[a] (2.3)	58.5[ab] (7.5)	ND	9.9[b] (0.6)	20.8[bc] (10.4)
50 m*M*	9.8[a] (1.2)	61.2[a] (3.1)	ND	5.2[d] (0.2)	23.9[bc] (4.5)
100 m*M*	9.9[a] (3.2)	61.6[a] (0.7)	ND	3.1[e] (0.1)	25.5[bc] (2.6)
200 m*M*	9.7[a] (0.7)	60.4[ab] (2.7)	ND	2.7[e] (0.2)	27.3[bc] (3.5)
500 m*M*	10.8[a] (1.1)	65.2[a] (3.1)	ND	2.9[e] (0.1)	21.1[bc] (4.4)

[a–e]Means with different superscripts in the same column are significantly different ($P < 0.05$).

[1]Given values are mean values of duplicate trials, n = 2. Values in parentheses represent SD. Ovotransferrin in the control sample could not be determined due to overlapping of peaks.

[2]ND = below detection level.

the content of ovomucin was significantly decreased with the addition of KCl, which was in good agreement with a previous report that the KCl washing step caused loss of ovomucin (Robinson and Monsey, 1969); the lowest content of ovomucin was found at the lowest concentration of KCl tested (2.5 m*M*). With the exception of a marginal decrease in lysozyme content, the concentrations of other proteins were not affected by various KCl concentrations. Compared with NaCl and $CaCl_2$, adding KCl did not affect the protein composition of the precipitates. Protein-protein interaction is a really complex phenomenon; despite recent progress in computational methods, there are no molecularly based models that are able to describe the effects of the salt on protein-protein interactions in their full complexity. As a result, it is difficult to identify with certainty the physical origins of the trends observed experimentally (Dumetz et al., 2007).

Protein composition of ovomucin precipitates prepared using different concentrations of KCl is given in Table 3. There was significant difference in ovalbumin, lysozyme, and ovomucin content when compared with the control sample. Ovomucoid could not be detected by the gel filtration chromatography method. The content of ovalbumin in all KCl-treated samples was greater than 50%; the concentration of KCl did not affect the composition of various proteins in the precipitates. From the present study, it is clear that KCl is not an ideal salt for isoelectric precipitation of ovomucin. The presence of a high concentration of ovotransferrin, ovalbumin, and lysozyme is evidenced by the thicker bands at 78, 45,and 14 kDa, respectively (Figure 6). Consequently, the percentage purity of ovomucin is considerably less.

Yield of crude ovomucin prepared as a function of KCl concentrations varied from 69.3 to 138.5 mg/100 g of egg albumen, which corresponds to 16.0 to 31.9% of the total ovomucin present in the egg white (Table 4). These yields were found to be significantly lower than that of the control sample (260.4 mg/100 g of egg

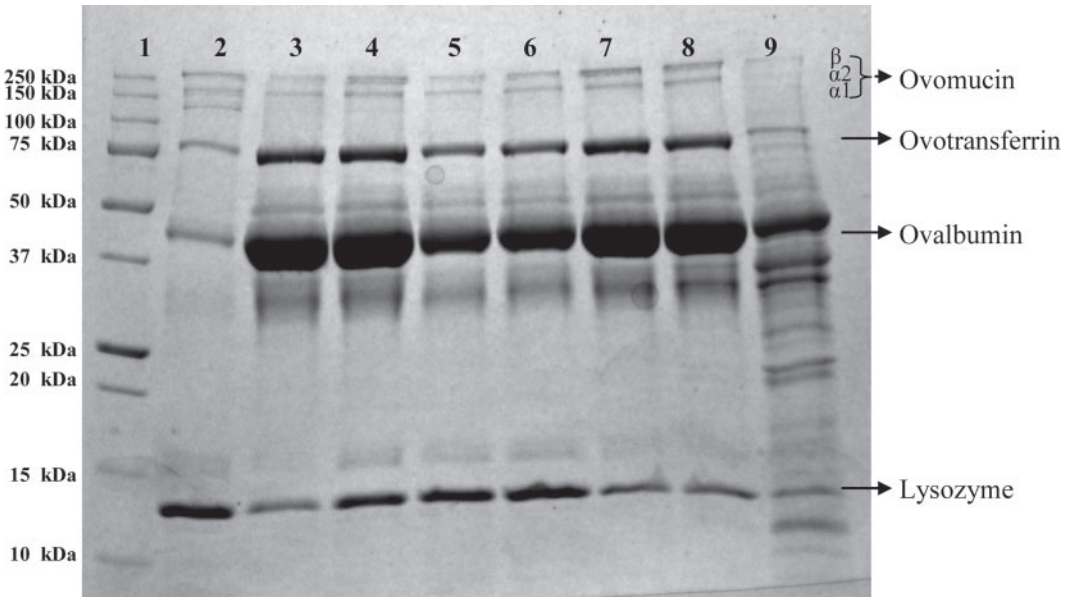

Figure 6. The SDS-PAGE pattern of proteins during ovomucin extraction using different KCl concentrations. Note that an equal concentration of samples was loaded. Lane 1 = standard molecular weight marker; lane 2 = control, 0 m*M*; lane 3 = 2.5 m*M*; lane 4 = 5 m*M*; lane 5 = 10 m*M*; lane 6 = 50 m*M*; lane 7 = 100 m*M*; lane 8 = 200 m*M*; lane 9 = 500 m*M*.

Table 4. Yield of ovomucin prepared in the presence of KCl[1]

Treatment	Ovomucin yield expressed as mg/100 g of egg albumen	Ovomucin yield expressed as percentage of ovomucin in egg white, %
Control, 0 m*M*	260.4[a] (1.2)	64.8[a] (0.2)
2.5 m*M*	69.3[e] (3.1)	16.0[e] (0.7)
5 m*M*	127.0[c] (2.9)	29.2[c] (0.7)
10 m*M*	138.5[b] (3.3)	31.9[b] (0.8)
50 m*M*	111.1[d] (2.8)	25.6[d] (0.6)
100 m*M*	129.1[c] (1.9)	29.7[c] (0.4)
200 m*M*	132.0[bc] (2.4)	30.4[bc] (0.6)
500 m*M*	105.4[d] (3.2)	24.3[d] (0.7)

[a–e]Means with different superscripts in the same column are significantly different ($P < 0.05$).

[1]Given values are mean values of duplicate trials, n = 2. Values in parentheses represent SD.

albumen). Brooks and Hale (1961) reported a yield of 90 to 117 mg/100 g of albumin after repeated washings with water and 2% KCl solution; KCl washings were reported to cause significant loss of ovomucin by Lyndrup (1973).

Ovomucin usually occur as a complex with lysozyme and other proteins in the egg white (Cotterill and Winter, 1955; Kato et al., 1976). High ionic environment weakens the electrostatic interaction among proteins. The interaction between lysozyme and ovomucin is mainly electrostatic in nature; in general, lysozyme is less precipitated at high concentrations of salt solutions.

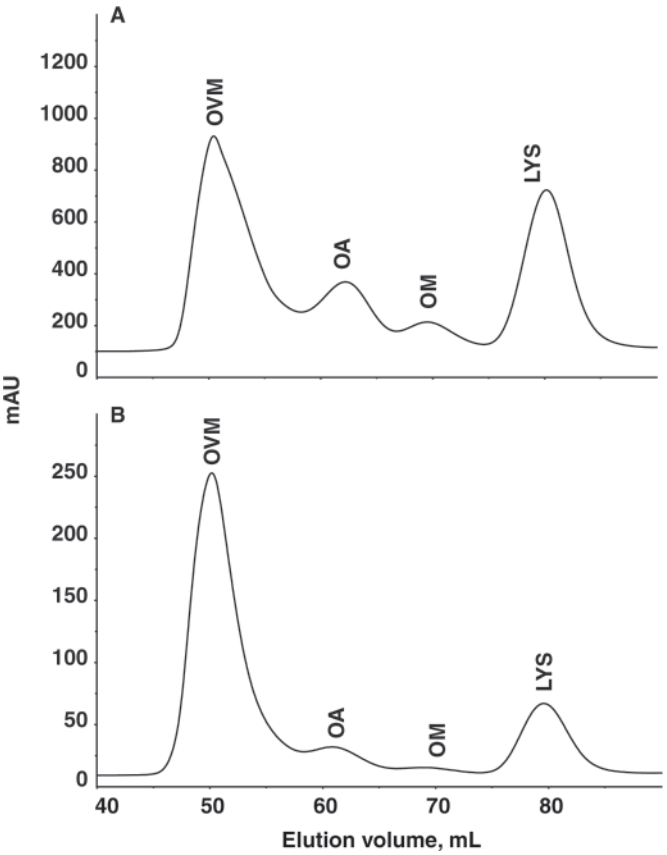

Figure 7. Gel filtration chromatogram of ovomucin prepared by (A) the conventional method and (B) the 2-step method of precipitation using 50 m*M* followed by 500 m*M* $CaCl_2$ solution. OVM = ovomucin; OA = ovalbumin; OM = ovomucoid; LYS = lysozyme.

Magnesium ions and NaCl were reported to decrease the interaction between ovomucin and lysozyme (Robinson, 1972). The present study revealed that $CaCl_2$, but not KCl, also behaves in the same manner.

Ovomucin Prepared by Precipitation with 50 mM $CaCl_2$ Followed by 500 mM $CaCl_2$ Solution

Preparation of ovomucin as a function of $CaCl_2$ concentration revealed a maximum purity of ovomucin at 50 m*M* concentration, in which both concentrations of ovalbumin and lysozyme were moderate (ovalbumin – 11.5% and lysozyme – 19.0%). Hence, an attempt has been made to precipitate the egg white proteins, first with 50 m*M* $CaCl_2$ solution followed by treating the precipitate with 500 m*M* $CaCl_2$ solution as per the protocol given in Figure 1. The purity of ovomucin prepared by the 2-step method was analyzed by gel filtration chromatography (Figure 7B). Gel filtration chromatogram showed only 1 major peak correspond-

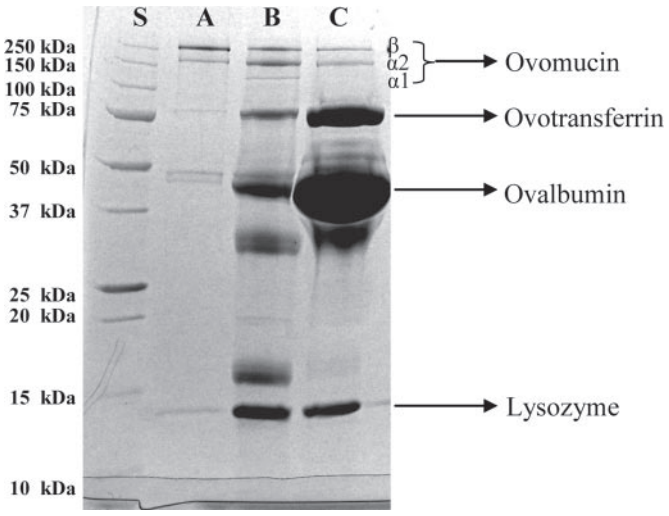

Figure 8. The SDS-PAGE pattern of ovomucin prepared under various conditions; egg white has been given as reference. S = standard marker; A = precipitation with 50 m*M* $CaCl_2$ followed by 500 m*M* $CaCl_2$; B = conventional ovomucin; C = freeze-dried egg white protein.

ing to ovomucin, with 2 minor peaks corresponding to ovalbumin and lysozyme, respectively. The purity of the ovomucin was calculated to be 97.3%, and the contents of ovalbumin, lysozyme, and ovomucoid were 1.3, 1.1, and 0.4%, respectively. Gel filtration chromatogram of ovomucin prepared using the conventional method is given (Figure 7A) for comparison. Contamination of ovomucin prepared by conventional method is evident from peaks corresponding to ovalbumin, ovomucoid, and lysozyme. The purity of the ovomucin prepared by conventional method was 66.8% due to high concentrations of ovalbumin (14.8%), ovomucoid (8.9%), and lysozyme (9.5%).

Yield of crude ovomucin prepared using the 2-step method was 410.1 mg/100 g of egg white, whereas that of ovomucin prepared using the conventional method was 185.5 mg/100 g of egg albumen. The difference in yield of ovomucin prepared by different researchers (Brooks and Hale, 1961; Lyndrup, 1973; Toussant and Latshaw, 1999) may be attributed to the difference in the methods of preparations. The yield obtained by the 2-step method using NaCl solution was 400.2 mg/100 g of egg albumen (Omana and Wu, 2009).

The purity of the prepared ovomucin was further analyzed by SDS-PAGE. The SDS-PAGE revealed strong bands just above 250 and 1 band between 250 and 150 kDa, which corresponds to ovomucin subunits (Figure 8). The faint bands were ovalbumin (45 kDa) and lysozyme (14.5 kDa), which further confirms the gel filtration results. The bands in the present study are closely related to that of β and α2 subunits of ovomucin as suggested by Itoh et al. (1987). The third subunit, α1 subunit, might be washed away during the second step using a high concentration of $CaCl_2$ (500 m*M*). At high concentrations of $CaCl_2$, α1 subunit was found to be disappearing (Figure 4).

Our results show that KCl is not an efficient salt in purifying ovomucin although it was widely used in literature as the last step of washing; $CaCl_2$ was found to be effective in preparing high-purity ovomucin (greater than 90%) at mild conditions. Ovomucin has been suggested to have vast potential to be developed as a functional food and nutraceutical ingredient. The development of a method for preparing highly pure ovomucin will facilitate our further work, which is under way to explore this great potential. Moreover, the ready availability of high-purity ovomucin would also facilitate our understanding of the structure and functional property relationship of this unique protein, which is poorly characterized in many ways.

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