Separation of Ovomucin Subunits by Gel Filtration: Enhanced Resolution of Subunits by Using a Dual-Column System

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An improved procedure involving a dual-column gel-filtration system for the separation of ovomucin subunits is described. Ovomucin, prepared from thick egg albumin by precipitation, was separated in the presence of sodium dodecyl sulfate (SDS) and β-mercaptoethanol by using two Sephacryl S-400 HR gel-filtration columns connected in series. The separation resulted in the isolation of eight peaks. The amino acid compositions and SDS−polyacrylamide gradient gel electrophoretic pattern of each peak were determined.

Keywords: *Ovomucin; subunits; gel filtration; molecular weight; amino acid composition*

INTRODUCTION

Ovomucin is an egg white protein representing about 2−4% of the total egg albumin protein. It is characterized by high molecular weight, high carbohydrate content, and a subunit structure. In the avian eggs, ovomucin is responsible for the gellike properties of thick egg albumin, and degradation of ovomucin complex is generally accepted as a reason for egg white thinning (Kato et al., 1971; Robinson and Monsey, 1972).

Ovomucin can be easily fractionated from egg albumin by dilution of egg albumin with water followed by acidification. The gelatinous ovomucin precipitate thus obtained is, however, contaminated with other egg albumin proteins, and, therefore, it is usually repeatedly washed with water and 2% KCl (Brooks and Hale, 1959; Kato et al., 1970). The crude ovomucin prepared in this way is highly insoluble in common buffer solutions and can be solubilized completely only with buffers containing dissociating agents such as urea, guanidine hydrochloride, or SDS as well as reducing agents such as β-mercaptoethanol or dithiothreitol (Robinson and Monsey, 1971; Itoh et al., 1987). The crude ovomucin treated in this manner has been shown to be composed of carbohydrate-rich (β-ovomucin) and carbohydrate-poor (α-ovomucin) subunits (Kato et al., 1971; Robinson and Monsey, 1971). More recently, Itoh et al. (1987) reported that the α-ovomucin component of crude ovomucin, which was fractioned by gel filtration, separated into two bands on SDS−PAGE. Itoh et al. (1987) named the components as α1- and α2-ovomucin. Similar SDS−PAGE patterns for reduced crude ovomucin have also been obtained both by Guérin and Brulé (1992) and by Guérin-Dubiard et al. (1993).

The aim of this work was to develop a gel-filtration method for an enhanced resolution among crude ovomucin subunits. Ovomucin, preparated from thick egg albumin by precipitation, was separated in the presence of SDS and β-mercaptoethanol by using two Sephacryl S-400 HR gel-filtration columns connected in series. The separation resulted in the isolation of eight peaks. The fractions obtained were analyzed by SDS−polyacrylamide gradient gel electrophoresis, and their amino acid compositions were also determined.

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MATERIALS AND METHODS

Preparation of Ovomucin. Ovomucin was preparated by means of the method developed by Kato et al. (1970) with a few modifications. Briefly, thick egg albumin was separated from total egg albumin by using a sieve and homogenized by using a household mixer at low speed to avoid foaming. The homogenized thick albumin was diluted with three volumes of deionized water, stirred for 10 min, and then adjusted to pH 6 with 1 N HCl. The thick albumin mixture was centrifuged (10 000*g*, 10 min at room temperature) to precipitate the crude ovomucin. The ovomucin precipitate was washed twice with water by centrifugation and then lyophilized.

Gel Filtration. Gel filtration was performed with an FPLC system (Pharmacia, Uppsala, Sweden; FPLC, Sephacryl, PhastSystem, and PhastGel are trademarks owned by Pharmacia) at room temperature. Ovomucin (5 mg) was dissolved in 10 mL of 150 mM imidazole hydrochloride buffer (pH 7.0) containing SDS (50 mg/mL) and β-mercaptoethanol (β-ME, 10 μL/mL) by overnight stirring. The dissolved ovomucin samples were boiled for 5 min to dissociate proteins into their subunits. Samples were centrifuged (10 000*g*, 10 min at room temperature), and 2 mL of ovomucin sample was applied to two Sephacryl S-400 HR columns (2.6 × 95 cm and 1.6 × 90 cm) connected to series. This dual-column system was eluted with 150 mM imidazole hydrochloride (pH 7.0), containing SDS (5 mg/mL) and β-ME (2 μL/mL), at a flow rate of 0.4 mL/min. The eluate was monitored by a UV detector at 280 nm and collected in 3 mL fractions. Three or four fractions from the middle of each peak were pooled, dialyzed against distilled water, and then lyophilized.

SDS−Polyacrylamide Gradient Gel Electrophoresis. SDS−PGGE was performed by PhastSystem using PhastGel gradient 4−15 minigels and PhastGel SDS buffer strips (Pharmacia, Uppsala, Sweden). The protein samples for SDS−PGGE were diluted 1:1 with 10 mM Tris-HCl−1 mM EDTA buffer (pH 8.0), and 1 μL of sample per lane was applied on the gel. The separation conditions were as follows: 250 V, 10 mA, 3.0 W, 15 °C for 75 Vh. After electrophoresis, the gel was stained in the development unit of PhastSystem by the silver nitrate method described in PhastSystem development technique file 210. The HMW−SDS kit (Pharmacia, Uppsala, Sweden) was used for molecular weight (MW) estimations.

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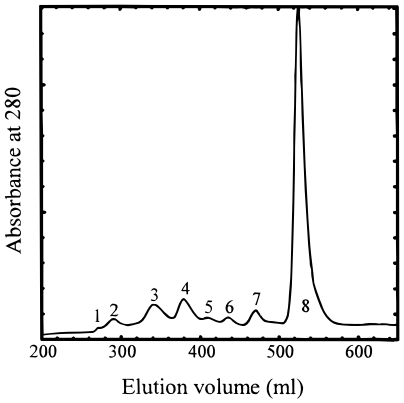

Figure 1. Elution profile of crude ovomucin obtained by Sephacryl S-400 HR gel-filtration chromatography. Analysis conditions were as described in Materials and Methods.

Removal of SDS. Prior to amino acid analyses, the lyophilized protein samples were freed of SDS by the ion-pair extraction method of Koningsberg and Henderson (1983). The extraction solvent consisted of anhydrous acetone, triethylamine, acetic acid, and water (85:5:5:5 v/v/v/v).

Amino Acid Analysis. Amino acids were analyzed as phenylthiocarbamate derivatives by HPLC (Millipore Corporation, 1987). Corrections for the destruction of amino acids during hydrolysis were not made, and tryptophan was not determined. *N*-Acetylglucosamine was coeluted with serine.

RESULTS AND DISCUSSION

The gel-filtration method developed in this work differed in some respects from methods used by other groups and the major differences will be briefly discussed. To maximize the yield of crude ovomucin preparation, we omitted the 2% KCl washing steps usually applied in crude ovomucin preparation, because it has been reported that KCl extracts also contain ovomucin (Robinson and Monsey, 1969; Lyndrup, 1973). Basically, we used the same sample and run buffers as Itoh et al. (1987), with the exception that the molarity of imidazole buffer used in this work was 150 mM instead of 50 mM. It has been recommended that the ionic strength of an eluent buffer should be at least 150 mM for Sephacryl HR to prevent possible ionic interactions with the gel matrix (Pharmacia Biotech, 1993). Furthermore, after overnight stirring, the dissolved ovomucin samples were boiled for 5 min to confirm the dissociation of proteins into their subunits. Moreover, to obtain good resolution, the sample volume, sample concentration, and eluent flow rate were kept relative low. Finally, two Sephacryl S-400 HR columns were connected in series, because our preliminary studies (data not shown) with one Sephacryl S-400 HR column showed insufficient resolution between high molecular weight compounds. A typical gel-filtration pattern of the reduced thick white crude ovomucin obtained by using our Sephacryl S-400 HR dual-column system is shown in Figure 1. The crude ovomucin was eluated into eight peaks, and the resolution, especially within the high-molecular-weight compounds, is far superior than ever reported before. According to SDS−PGGE performed with egg albumin protein standards (data not shown), peak 6 was identified as ovotransferrin, peak 7 as ovalbumin, and peak 8 as lysozyme. The characteristics of peaks 1−5 will be discussed in detail.

In this study, a PhastGel gradient 4−15 minigel was used in the protein MW estimation studies. Lambin et al. (1976) demostrated that, by using SDS−PGGE, there

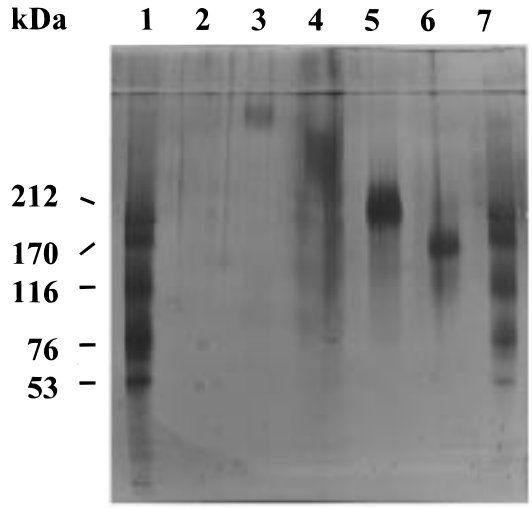

Figure 2. SDS−PGGE (4−15%) of pooled fractions from gel filtration. (Lane 1) Molecular weight markers; (lane 2) peak 1; (lane 3) peak 2; (lane 4) peak 3; (lane 5) peak 4; (lane 6) peak 5; (lane 7) molecular weight markers.

is a linear relationship between the log MW and the log of the polyacrylamide concentration (T) reached by a protein. This relationship can be written as log MW = a log $T + b$, where a and b are the slope and intercept, respectively, of the linear regression. This relationship, in combination with SDS−PGGE, permits the estimation of MW over a much wider range than a linear regression between the log MW of a protein and its relative mobility (R_f) and also provides more accurate MW estimations for glycoproteins (Lambin et al., 1976; Lambin, 1978; Poduslo, 1981). Therefore this technique appears to be quite suitable for MW estimations of ovomucin subunits, which are high molecular weight glycoproteins.

An SDS−PGGE diagram of proteins obtained from gel filtration (peaks 1−5) is shown in Figure 2. Because protein(s) corresponding to peak 1 was(were) poorly detected, the molecular weight could not be estimated by SDS−PGGE. However, peak 1 must contain a very high molecular weight compound(s) since it eluted in the void volume of Sephacryl S-400 HR gel-filtration column (Figure 1). The exclusion limit of Sephacryl S-400 HR gel has been estimated to be ≥2 000 000 for dextrans and ≥8 000 000 for globular proteins (Pharmacia Biotech, 1993) and according to Reynolds and Tanford (1970), protein−SDS complexes appeared as rodlike particles. Therefore, it is likely that protein eluted in peak 1 has a molecular weight of over 2 000 000. In contrast to peak 1, all other four peaks were clearly detected in SDS−PGGE (Figure 2) and separated as single protein bands. Only additional faint bands of other albumin proteins as contaminants were observed. The MWs of peaks 2−5 were determined by the technique presented by Lambin et al. (1976). In this study, the relationship obtained was log MW = −3.47 log T + 8.42 (R = 0.994) by using the HMW−SDS kit as standard (lane 1 in Figure 2), and MWs of 610 000, 350 000, 210 000, and 160 000 were estimated for proteins in peaks 2, 3, 4, and 5, respectively.

According to the gel-filtration pattern (Figure 1), peak 1 contained protein(s) having a molecular weight ≥2 000 000. To our knowledge, ovomucin is the only egg white protein having a molecular weight as high as this. However, the amino acid composition of protein(s) in peak 1 was different from the amino acid compositions

Table 1. Amino Acid Composition of Protein Peak 1 Obtained by Sephacryl S-400 HR Gel Filtration: Comparison with Previously Published Data for Ovomucin Complex

amino acid[a]	peak 1	Robinson and Monsey (1971)[b]	Adachi et al. (1973)[b]	Sleight et al. (1973)[c]	Sleight et al. (1973)[d]	Sleight et al. (1973)[b]	Sleight et al. (1973)[b,e]	Smith et al. (1973)[b]	Smith et al. (1973)[b]	Smith et al. (1973)[b,f]	Robinson and Monsey (1975)[d]	Sato and Hayakawa (1977)[d]
Ala	5.4	4.7	6.1	4.5	4.6	4.9	5.6	ND	4.5	4.6	5.9	7.3
Arg	3.3	3.1	3.4	2.9	3.4	2.9	1.8	3.3	3.2	3.4	4.2	3.4
Asp	8.5	9.1	11.1	10.0	11.0	11.3	11.8	11.8	10.5	11.1	9.0	10.8
Cys	0.7	7.2	6.7	7.9	7.8	7.0	8.3	ND	7.3	8.1	4.1	6.0
Glu	13.6	10.2	9.2	10.0	10.8	11.5	8.7	11.6	11.5	10.4	9.0	0.4
Gly	13.5	5.9	8.4	6.4	6.8	7.0	7.2	6.9	6.3	6.1	6.0	7.3
His	1.2	2.3	1.9	2.1	2.0	2.0	1.3	2.2	2.2	1.9	1.5	1.8
Ile	4.4	4.3	5.6	4.6	4.6	5.5	5.2	4.3	4.7	4.8	3.8	5.8
Leu	8.1	6.7	6.1	5.8	6.1	6.3	6.8	6.3	6.6	6.4	7.5	8.4
Lys	4.4	6.3	6.5	6.1	5.6	6.0	4.1	6.7	6.0	6.4	5.2	6.6
Met	1.4	2.0	1.6	1.6	1.6	1.8	1.9	1.9	1.6	1.5	1.9	1.7
Phe	2.9	3.9	5.0	3.9	4.0	4.1	4.8	4.3	4.2	4.2	3.4	4.1
Pro	5.6	6.4	2.2	5.1	5.8	4.6	5.7	6.3	6.3	5.6	7.0	4.8
Ser	12.8	9.3	9.0	7.6	7.6	4.8	7.1	8.8	7.8	7.8	12.0	8.2
Thr	6.7	8.4	6.4	7.5	7.2	6.3	7.2	8.0	7.7	7.7	11.0	7.2
Trp	ND	0.8	ND	3.6	1.2	1.8	1.6	ND	ND	ND	ND	ND
Tyr	2.6	2.9	3.3	3.2	3.4	3.6	3.7	3.3	3.5	3.5	2.7	3.5
Val	5.1	6.2	7.6	6.3	6.4	7.6	7.3	5.9	6.1	6.5	5.9	5.7

[a] Values are in moles per mole of residues. [b] Ovomucin complex from whole thick albumin. [c] Insoluble ovomucin complex from thick albumin. [d] Soluble ovomucin complex from thick albumin. [e] Ovomucin separated by using the method of Donovan et al. (1970). [f] Reduced and alkylated.

Table 2. Amino Acid Composition of Protein Peak 2 Obtained by Sephacryl S-400 HR Gel Filtration: Comparison with Previously Published Data for the Thick Albumin *β*-Ovomucin

amino acid[a]	peak 2	Robinson and Monsey (1971)	Robinson and Monsey (1975)	Itoh et al. (1987)	Tsuge et al. (1997)
Ala	4.9	6.4	5.3	5.0	7.7
Arg	2.5	2.6	2.3	2.6	1.6
Asp	5.0	7.2	4.9	5.9	4.8
Cys	2.0	5.4	3.6	3.0	1.4
Glu	6.8	8.6	7.1	8.2	7.9
Gly	4.4	4.6	3.8	4.3	3.2
His	1.8	1.7	1.8	2.1	1.1
Ile	3.7	3.9	3.7	3.9	4.1
Leu	6.8	6.9	7.0	7.0	7.3
Lys	4.3	5.1	5.4	6.1	4.6
Met	2.1	1.4	1.5	1.6	4.4
Phe	2.7	3.0	2.7	3.8	2.6
Pro	8.9	7.4	9.1	8.7	9.0
Ser	25.2	14.3	17.1	15.9	18.9
Thr	14.6	13.3	17.2	14.3	14.8
Trp	ND	0.8	1.2	ND	ND
Tyr	2.7	2.5	2.4	2.6	1.7
Val	4.6	4.9	4.9	5.1	4.9

[a] Values are in moles per mole of residues.

Table 3. Amino Acid Compositions of Protein Peaks 3 and 4 Obtained by Sephacryl S-400 HR Gel Filtration: Comparison with Previously Published Data for the Thick Albumin α-Ovomucin

amino acid[a]	peak 3	peak 4	Robinson and Monsey (1971)	Itoh et al. (1987)[b]	Tsuge et al. (1997)
Ala	5.3	5.1	4.8	5.1	4.8
Arg	3.0	3.1	2.7	3.2	2.3
Asp	10.7	11.1	9.9	10.1	11.9
Cys	5.6	6.8	8.7	7.0	5.2
Glu	11.1	11.0	11.4	10.6	10.9
Gly	8.5	8.8	6.8	7.2	7.4
His	2.6	2.7	2.0	2.2	2.1
Ile	5.0	5.1	4.6	5.0	5.2
Leu	6.1	6.2	6.4	6.3	6.2
Lys	5.4	ND	5.5	6.5	6.4
Met	1.8	1.8	0.8	1.9	2.2
Phe	4.2	4.5	4.4	4.5	4.0
Pro	6.3	6.2	5.4	5.8	5.9
Ser	10.4	10.1	8.1	7.8	7.7
Thr	7.4	7.5	7.5	7.2	7.8
Trp	ND	ND	1.3	ND	ND
Tyr	3.5	3.5	3.4	3.8	3.5
Val	6.5	6.6	6.1	5.8	6.6

[a] Values are in moles per mol of residues. [b] α-Ovomucin component that contained both α1- and α2-ovomucin.

reported previously for the thick albumin ovomucins (Table 1). Therefore, it was concluded that our high molecular weight protein was not an unreduced residue of ovomucin complex. The amino acid composition of the peak 1 component was also different from the amino acid compositions of peaks 2−5 (Tables 2−4). It was therefore also evident that peak 1 was not an oligomeric form of any of the proteins corresponding to peaks 2−5. However, the corresponding peak 1 respective to protein(s) may represent some kind of aggregated form of ovomucin subunits that may exist in the thick egg albumin gel. It is interesting that Rabouille et al. (1990) have reported a molecular weight of about 3 300 000 for ovomucin fragments after a reduction of ovomucin by dithiothreitol. Unfortunately, they did not report the amino acid composition for this high molecular weight-reduced ovomucin. Therefore, this amino acid composition for protein in peak 1 (Table 1) could potentially be the first ever composition reported for a high-molecular-weight ovomucin fragment isolated from thick albumin after reduction.

The amino acid composition of peak 2 was comparable to those reported for *β*-ovomucin previously (Table 2). The much higher quantity of serine in our preparation is due to coeluting *N*-acetylglucosamine. It is noteworthy that peak 2 was stained yellowish with silver nitrate in SDS−PGGE, in contrast to other bands which were stained dark brown (Figure 2). Highly glycosylated proteins are known to stain weakly by silver staining protocols, probably because of steric interference (Jay et al. 1990). Previous studies have also indicated that thick albumin *β*-ovomucin is a highly glycosylated protein, having a carbohydrate content of about 50% (Robinson and Monsey, 1971; Itoh et al., 1987). The molecular weight, 610 000—estimated in this study for protein in peak 2—is within the range (523 000−743 000) reported by Robinson and Monsey (1975) for

Table 4. Amino Acid Composition of Protein Peak 5 Obtained by Sephacryl S-400 HR Gel Filtration: Comparison with Previously Published Data for Ovostatin

amino acid[a]	peak 5	Miller and Feeney (1966)	Donovan et al. (1969)	amino acid[a]	peak 5	Miller and Feeney (1966)	Donovan et al. (1969)
Ala	7.2	5.8	5.8	Lys	5.1	6.0	5.7
Arg	3.5	3.8	3.6	Met	2.4	2.1	2.0
Asp	9.1	9.5	10.2	Phe	4.8	5.1	4.8
Cys	0.9	1.7	1.8	Pro	5.2	5.3	4.3
Glu	11.7	11.5	11.6	Ser	9.7	7.4	8.0
Gly	8.7	5.1	5.1	Thr	6.6	6.7	0.4
His	2.1	1.8	1.8	Trp	ND	0.7	1.2
Ile	4.7	6.5	6.5	Tyr	3.1	3.9	3.9
Leu	8.2	9.2	9.1	Val	7.2	7.9	8.2

[a] Values are in moles per mole of residues.

β-ovomucin but is in contrast to the 400 000 reported by Hayakawa and Sato (1978), Itoh et al. (1987), and Tsuge et al. (1996, 1997). Summarizing these data, it is quite obvious that peak 2 contained a polypeptide corresponding to β-ovomucin.

When the amino acid compositions of peak 3 and 4 were compared, no essential differences were observed (Table 3). The amino acid compositions of peaks 3 and 4 corresponded to that of α-ovomucin as reported both by Robinson and Monsey (1971) and by Tsuge et al. (1997) and, moreover, to the α-component (α1- and α2-ovomucin) by Itoh et al. (1987). The higher serine contents in our fractions are due to coeluting *N*-acetylglucosamine, and the lower cysteine content is caused by its destruction during acid hydrolysis. The molecular weight of α-ovomucin was estimated to be 210 000 by Robinson and Monsey (1971) and 220 000 by Tsuge et al. (1997). However, Itoh et al. (1987) estimated the molecular weights 150 000 and 220 000 for α1- and α2-ovomucin, respectively, whereas in our study peak 3 was found to have a molecular weight of 350 000 and, with peak 4, about 210 000. Regarding these findings, it is likely that peak 4 equals α2-ovomucin of Itoh et al. (1987) as well as the α-ovomucin subunit reported by both Robinson and Monsey (1971) and Tsuge et al. (1997). However, peak 3 appears to be a possible new α-ovomucin subunit that has not managed to fractionate before. The molecular weight difference between the compounds in peaks 3 and 4 might be caused by a different degree of glycosylation between these two proteins. The self-association of α-ovomucin in dilute salt solutions has been previously reported by several researchers (Robinson and Monsey, 1971; Hayakawa and Sato, 1978; Miller et al., 1982). Therefore, it could also be possible that peak 4 is a α-ovomucin monomer and peak 3 corresponds to the dimeric form of α-ovomucin, as held together by specific hydrogen bonds between carbohydrate moieties free of SDS.

Peak 5 has a similar molecular weight to the α1-ovomucin of Itoh et al. (1987). However, the amino acid composition of peak 5 showed some differences in comparison to that of peaks 3 and 4, especially in the quantity of cysteine (Table 4). Due to the low amount of cysteine in peak 5, it may be that the protein in peak 5 is not α-ovomucin at all. The amino acid composition of peak 5 (Table 4) was quite similar to that of ovostatin as previously reported by Miller and Feeney (1966) and Donovan et al. (1969). Moreover, Nagase et al. (1983) have reported that ovostatin is composed of four similar subunits having a molecular weight of 165 000; a molecular weight of 160 000 was estimated in this study for peak 5. Also, Awadé et al. (1994) and Vachier et al. (1995) have suggested that ovomucin preparations might contain ovostatin as a contaminant. Therefore, it is quite possible that the protein in peak 5 (Figure 1) is ovostatin rather than α-ovomucin.

In conclusion, our dual-column system reveals the best resolution reported so far between high-molecular-weight compounds of reduced crude ovomucin, and it also indicates the complicated nature of ovomucin complex. However, further studies for more accurate identification of peaks 1−5, e.g., carbohydrate content determination, are needed.

ABBREVIATIONS USED

β-ME, β-mercaptoethanol; SDS−PGGE, sodium dodecyl sulfate−polyacrylamide gradient gel electrophoresis.

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