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Comparison of Different Egg Albumen Fractions as Sources of Ovomucin

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Ovomucin was fractionated from whole egg albumen, thick egg albumen, liquid egg albumen, and a liquid egg albumen filtration byproduct by using the isoelectric precipitation method. The amounts of ovomucin measured in the above-mentioned fractions were 280, 340, 500, and 520 mg per 100 g of albumen, respectively. There was great variation between the β-ovomucin contents of the different albumen fractions. Whole egg albumen contained about 25 mg of β-ovomucin in 100 g of albumen, whereas thick egg albumen, liquid egg albumen, and the filtration byproduct contained about 1.5, 3, and 5 times more β-ovomucin, respectively, as compared to whole egg albumen. The results indicate that both the liquid egg albumen fraction and especially the filtration byproduct fraction appear to be potential sources of ovomucin when it is used as an ingredient for functional foods.

KEYWORDS: Ovomucin; functional foods; egg albumen; fractionation

INTRODUCTION

The views and expectations of western consumers concerning food have changed radically within the past few years. Traditionally, consumers have expected food to be safe, nutritious, and tasty. Today, however, they are increasingly paying attention also to the health aspects of foods: food products are expected to maintain good health and prevent diseases. This has increased the need to develop health-promoting or so-called functional foods. There is, thus, a definite need to explore food compounds with biological activities—for example, glycoproteins. Glycoproteins are macromolecules that have one or more carbohydrate chains, glycans, linked to a peptide chain. The share of carbohydrates in glycoproteins varies between 1 and 80% (*1*). Glycoproteins are biologically very active and are involved in numerous biological cell functions, including structural, barrier, reproduction, transport, protection, and immunological functions (*2*). One rich source of glycoproteins is the albumen of the hen's egg, which contains various glycoproteins such as ovalbumin, ovotransferrin, ovomucoid, and ovomucin (*3*, *4*). Ovomucin is a particularly interesting protein, because it has been reported to have antiviral and antitumor properties (*5*−*13*).

Ovomucin can be extracted and purified from egg white by isoelectric precipitation (IEP) (*14*−*18*) as well as by gel filtration (*19*−*25*). Although many studies have been conducted to elucidate the physical and chemical properties of ovomucin, ovomucin is still in many ways ill-defined. Moreover, there are considerable variations in the results of different laboratories. This is no doubt due to the heterogeneous nature of ovomucin as well as its poor solubility after isolation, but also, the different methods used in isolation and analysis methods give a lot of variation.

The aim of the present study was to compare different egg white fractions as sources of ovomucin. The egg white fractions for this study were selected on the basis of their potential bioactivity. We used the same methods for the separation and analysis of the different ovomucins in order to obtain comparable data, which may be of importance when ovomucin is utilized as an ingredient for functional foods. Whole egg albumen obtained from fresh shell eggs was chosen as a reference to which the results of the other albumen fractions were compared. Thick egg albumen was also included because many studies of ovomucin's biological activities have been carried out with ovomucin isolated from thick albumen. In egg processing plants, moreover, the chalazae are filtered off from egg albumen after breaking and yolk separation prior to further liquid egg albumen processing. This albumen fraction, referred to here as the filtration byproduct, and the liquid egg albumen fraction are the two other albumen fractions examined in this paper.

MATERIALS AND METHODS

Materials. The eggs used in the whole and thick egg white fraction studies were obtained from the local hen house (MTT Agrifood Research Finland, Animal Feeding Section). The liquid egg white and the filtration byproduct fraction were obtained as a gift by Scanegg Suomi Oy (Piispanristi, Finland). The egg albumen protein standards, ovalbumin (A-5503, purity 98%), ovotransferrin (C-0755, purity 98%), ovomucoid (T-2011), and lysozyme (L-6876, purity 95%), were purchased from Sigma (St. Louis, MO). Additionally, galactose (48 259,

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purity ≥ 99.5%), *N*-acetylglucosamine (01 140, purity ≥ 99.0%), and sialic acid (01 398, purity ≥ 99.0%) were purchased from Fluka (Buchs, Switzerland).

Separation of Egg White Fractions. The whole egg white fraction was obtained by separating the albumen and yolk with a household yolk separator. Whole egg whites (chalazae cords removed by using tweezers) of 15 eggs were pooled and homogenized with a household mixer at low speed to avoid foaming. Three samples (50 mL) were taken, and ovomucin was isolated from these as described below.

Thick and thin albumen were fractionated using a modified method of Holst and Almquist (*26*) and Brooks and Hale (*27*). Albumen, from which the yolk and chalazae cords had been removed, was placed on a sieve (width of square aperture of the sieve was 2.0 mm), which was laid onto a disposable Petri dish (i.d. 13 cm). The thick albumen was cut into several pieces with scissors to allow the inner thin white also to drain through the sieve. The outer and inner thin whites were allowed to pass through the sieve for 2 min, and the albumen remaining on the sieve was referred to as thick albumen or thick white. The thick white fractions of 15 eggs were pooled and homogenized with a household mixer at low speed to avoid foaming, and three samples (50 mL) were taken from which ovomucin was isolated (see below).

Three samples (50 mL) of liquid egg white were taken for ovomucin preparation. In addition, two samples per day (1 L) of the filtration byproduct fraction were taken at two consecutive days during liquid egg processing. Three 50 mL aliquots of every 1 L sample were further taken for ovomucin preparation.

Isolation of Ovomucin. Ovomucin was isolated from the egg white fractions by the IEP method developed by Kato et al. (*16*) but with a few modifications. The homogenized albumen was diluted with three volumes of deionized water, stirred for 30 min, and then adjusted to pH 6 with 1 N HCl. The albumen mixture was centrifuged (10 000*g*, 10 min at room temperature) to precipitate the crude ovomucin. The crude ovomucin precipitate was washed twice with water by centrifugation and then freeze-dried.

The freeze-dried ovomucin preparations were weighed, and the obtained values were used to calculate the crude ovomucin contents of the albumen fractions in 100 g of albumen and as percent of total protein. The total protein content of the albumen fractions was measured by the Kjeldahl method using a conversion factor of 6.25.

Gel Filtration Chromatography (GFC). The subunit profiles of the IEP ovomucins were determined with high-performance liquid chromatography (HPLC) equipment consisting of the following: pump (model 600E, Waters Chromatography Division, Milford, MA), dual wavelength detector (VWM2141, Amersham Biosciences, Uppsala, Sweden), and Nec data processing equipment (program Maxima, Waters). Lyophilized IEP ovomucin was dissolved in 100 mM of sodium phosphate buffer (pH 7.0) containing sodium dodecyl sulfate (SDS, 50 mg/mL) and β-mercaptoethanol (β-ME, 10 μL/mL) at concentrations of 5 mg/mL by overnight stirring. The samples were filtered through a GHP Acrodisc 0.45 μm syringe filter (Pall Gelman Laboratory, Ann Arbor, MI). Each sample (100 μL) was introduced to two Superose 6 HR 10/30 columns (Amersham Biosciences) connected to series and eluted with 100 mM of phosphate buffer (pH 7.0) containing SDS (5 mg/mL) and β-ME (1 μL/mL) at a flow rate of 0.2 mL/min. The eluate was monitored by a UV detector at 280 nm.

Carbohydrate Analysis of IEP Ovomucins. Total hexose content was determined by a resorcinol sulfuric acid micromethod (*28*). Ovomucin (2 mg) was hydrolyzed with 2 M HCl for 3 h at 95 °C. Galactose was used as a standard.

Total hexosamine was estimated by the 3-methyl-2-benzothiazoline hydrazone hydrochloride (MBTH) method using *N*-acetylglucosamine as a standard (*29*). Each sample (2 mg) was hydrolyzed with 2 M HCl for 3 h at 95 °C.

Sialic acid was determined by using a periodate−resorcinol method (*30*). Ovomucin (2 mg) was hydrolyzed with 0.01 M HCl for 1 h at 90 °C. *N*-Acetylneuraminic acid was used as a standard. It is known that free sialic acids are destroyed during the hydrolysis step. In this study, this loss was measured to be 12%, which was then corrected to reported values.

RESULTS AND DISCUSSION

Ovomucin was separated in this study by using a modified IEP method of Kato et al. (*15*) as described above. The KCl washing steps, which are generally applied in IEP methods, were omitted from this procedure for two reasons. First, we wanted to maximize the yield of ovomucin, and it has been found that KCl extracts contain ovomucin as well (*31*, *32*). Lyndrup (*32*) reported that 120 mg of ovomucin was obtained from 100 mL of thick albumen after an extensive washing procedure, while the KCl washings contained altogether 360 mg of ovomucin. Thus, only about 25% of the ovomucin present in thick albumen was obtained after vigorous washing. Second, we tried to keep the ovomucin separation procedure as simple as possible. It has been reported in previous publications that in order to achieve efficient removal of other coprecipitated albumen proteins, the ovomucin precipitate should be washed several times and/or kept in contact with water and KCl washing solutions for 24 h per washing step (*14*, *32*). Consequently, we included just two water washing steps into our purification protocol to remove only the major contaminants, thus leading to an ovomucin precipitate containing various amounts of other coprecipitated egg albumen proteins. These IEP ovomucin preparations are referred to here as crude ovomucins.

The purity of ovomucin preparations has usually been evaluated in earlier studies by electrophoretic methods, mainly SDS−polyacrylamide gel electrophoresis (PAGE). In this study, the purity of the different crude ovomucin preparations was examined by using GFC. The Superose 6 HR dual-column system applied by us separated the crude ovomucins into seven peaks (**Figure 1**). According to gel filtration performed with egg albumen protein standards (data not shown here), peak 4 was identified as ovotransferrin, peak 5 as ovalbumin, peak 6 as ovomucoid, and peak 7 as lysozyme. Peak 5 probably contained another or other egg albumen protein(s) as well, because in some preparations peak 5 seems to be fronting (**Figure 1A**,**B**) and in others it appears even to be separated into partially resolved peaks (**Figure 1C**,**D**). The nature of these proteins (this protein) was not further identified, but ovoinhibitor and ovoglobulins G2 and G3 are potential alternatives. A molecular weight of 47 000 has been reported for ovoglobulin G2, 49 000 for ovoinhibitor, and 50 000 for ovoglobulin G3, whereas the molecular weight attributed to ovalbumin is 45 000 (*4*). The peaks numbered 1−3 contained ovomucin subunits; peak 1 was named as β-ovomucin, peak 2 as α2-ovomucin, and peak 3 as α1-ovomucin, in accordance with the nomenclature of Itoh et al. (*21*).

The peak areas could not be used for purity estimations as such, since the absorbance for protein was measured at 280 nm. At this wavelength, the extinction coefficient for lysozyme is much higher as compared to the other coprecipitated proteins, which would have led to an overestimation of the amount of lysozyme in the crude ovomucin preparations. To avoid this, standard curves (peak area vs milligrams of protein) were created for each coprecipitated egg white protein (ovotransferrin, ovalbumin, ovomucoid, and lysozyme) by using commercial protein preparations and the Superose 6 HR dual-column gel filtration system. There was a linear relationship between the amount of protein (C) and the peak area (Y) with every standard protein. This relationship could be written as $C = aY$, where a is the slope of the linear regression when the curve was forced through zero. In this study, the relationship for ovotransferrin was $C = 3 \times 10^{-8}\ Y$ ($R = 0.9996$), for ovalbumin $C = 5 \times 10^{-8}\ Y$ ($R = 0.9986$), for ovomucoid $C = 1 \times 10^{-7}\ Y$ ($R = 0.9961$), and for lysozyme $C = 1 \times 10^{-8}\ Y$ ($R = 0.9996$). The

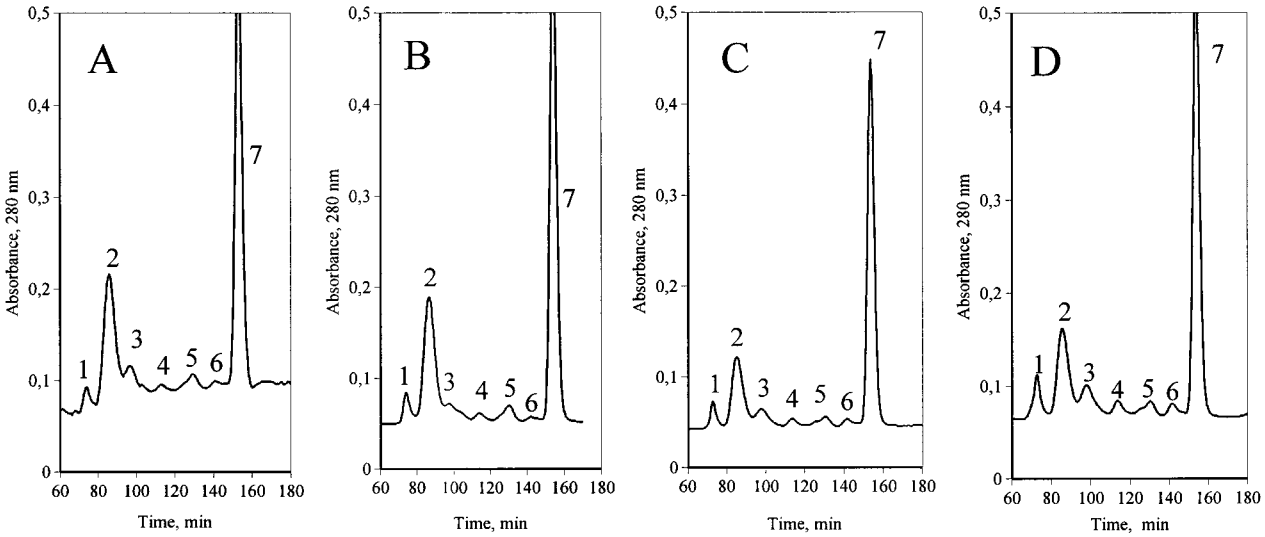

Figure 1. Elution profiles of crude ovomucins obtained by Superose 6 HR GFC. (A) Whole egg white, (B) thick egg white, (C) liquid egg white, and (D) filtration byproduct. Peak 1 = β-ovomucin, peak 2 = α2-ovomucin, peak 3 = α1-ovomucin, peak 4 = ovotransferrin, peak 5 = ovalbumin, peak 6 = ovomucoid, and peak 7 = lysozyme. Analysis conditions are described in Materials and Methods.

Table 1. Percentage Distribution of Coprecipitated Albumen Proteins and Ovomucin in Crude Ovomucin

egg white fraction	ovotransferrin (%)	ovalbumin (%)	ovomucoid (%)	lysozyme (%)	ovomucin (%)
whole egg white	1	7	4	24	64
thick egg white	1	6	2	26	65
liquid egg white	1	4	4	20	71
filtration byproduct	2	6	5	23	64

purity announced by the manufacturer for ovotransferrin, ovalbumin, and lysozyme was also take into account when generating the standard curves. In the case of ovomucoid, the manufacturer did not give the purity in percentage. Therefore, we analyzed a sample of ovomucoid by our gel filtration system and found that it contained lysozyme as an impurity. The amount of lysozyme was calculated by using the standard curve for lysozyme, and the purity of ovomucoid was estimated to be 99%. This value was then used to generate the standard curve for ovomucoid. Applying the values obtained for the coprecipitated albumen proteins by using above-mentioned standard curves and the known concentration of crude ovomucin in each gel filtration sample, we calculated the percentage distribution of coprecipitated albumen proteins in the different albumen fractions (**Table 1**). By subtracting the amounts of coprecipitated proteins in each crude ovomucin fraction, we found that the liquid egg white has the highest purity, namely, 71%, of the different fractions studied here. The purities of the other fractions were practically similar: 64% for the whole egg white and filtration byproduct fractions and 65% for the thick egg white fraction.

Using these purity factors, it was possible to estimate the actual ovomucin contents in the different albumen fractions. **Table 2** shows these corrected amounts concurrently with the crude ovomucin contents. Thus, 100 g of whole egg white contained 430 mg of crude ovomucin, corresponding to 4.2% of the total protein in egg white, whereas after correction whole egg white was found to contain 280 mg of ovomucin or 2.8% of the total protein. A review of the literature revealed great variance among previous studies: Brooks and Hale (*14*) reported that after exhaustive washing procedures whole egg albumen contained 90−117 mg of ovomucin per 100 g of albumen, while Toussant and Latshaw (*33*) quite recently found an ovomucin content of about 530 mg in 100 g of whole egg white. In

Table 2. Crude Ovomucin and Corrected Ovomucin Content

	crude ovomucin content		corrected ovomucin content	
egg white fraction	mg/100 g of albumen	% of total protein	mg/100 g of albumen	% of total protein
whole egg white	430	4.2	280	2.8
thick egg white	530	5.3	340	3.4
liquid egg white	710	6.8	500	4.8
filtration byproduct	820	7.8	520	5.0

addition, the values have been reported as follows: 350−370 (*34*), 310−360 (*35*), 425 (*15*), and 440 mg (*36*) of ovomucin in 100 g of whole egg white. It is clear that different hen hybrids as well as different purification and analysis methods for ovomucin also give some variation to the above-mentioned results, but in large part, this variation might be explained by the differences in the times of KCl washings. As already indicated in this paper, the degree of ovomucin precipitation decreases with a more exhaustive KCl washing procedure. Moreover, there are two kinds of ovomucins in egg white, namely, insoluble and soluble ovomucins. The thick white has been found to contain most of the insoluble ovomucin, in contrast to the thin white, which mainly contains soluble ovomucin. Thus, the lowest values (90−117 mg) for whole egg ovomucin reported by Brooks and Hale (*14*) after an exhaustive KCl washing procedure presumably corresponded to the amount of insoluble ovomucin in whole egg white, while the figure obtained by us, 280 mg, contained in addition to insoluble ovomucin also most of the soluble ovomucin present in egg white. Accordingly, the amount of crude ovomucin in this study (430 mg) and the higher values reported previously probably correspond to such ovomucin preparations that contained both insoluble and soluble ovomucins and, moreover, various amounts of coprecipitated egg white proteins. Furthermore, in this study,

Table 3. Carbohydrate Contents of Crude Ovomucins Obtained from Different Egg White Fractions[a]

	carbohydrate content					
egg white fraction	hexoses (% of ovomucin)	hexosamines (% of ovomucin)	sialic acid (% of ovomucin)	total (% of ovomucin)	total (mg/100 g of egg white)	refs
whole egg white	**6.9**	**13.9**	**2.8**	**23.6**	**100**	
	6.6	7.1	6.0	20.7		*38*
	5.5	7.8	1.9	14.8		*39*
	7.4	7.2	4.0	18.6		*15*
	8.1	9.5	1.2	18.8		*40*
thick egg white	**7.3**	**12.8**	**2.8**	**22.9**	**120**	
	11.3	12.6	7.1	31.0		*41*
	11.4	12.1	7.4	30.9		*20*
	10.7	10.1	7.4	28.2		*42*
	12.3	4.0	4.6	20.9		*43*
	7.3	8.4	3.8	19.5		*44*
	10.9	14.0	8.4	33.3		*45*
	13.1	14.3	7.3	34.7		*8*
liquid egg white	**5.5**	**12.9**	**2.1**	**20.5**	**145**	
filtration byproduct	**5.2**	**12.7**	**2.4**	**20.3**	**165**	

[a] The carbohydrate contents obtained in this study are presented in boldface.

we found that 100 g of thick egg white contained 530 mg of crude ovomucin, whereas the actual ovomucin content calculated for thick white ovomucin in 100 g of egg white was 340 mg. Again, as was the case with whole egg white ovomucin, the variation among the results of previous studies is very large. Baliga et al. (*37*) reported that thick egg albumen contained about 132 mg of ovomucin per 100 g of albumen, while the corresponding figure obtained by Toussant and Latshaw (*33*) was about 740 mg of ovomucin. Presumably, the reasons for these variations are the same as in the case of whole egg white ovomucin. On the other hand, we found only one study in which the ovomucin content for liquid egg white had been measured. Guérin and Brulé (*18*) obtained 4.7 g (per 100 g of total protein) of crude ovomucin from the liquid egg white fraction. This preparation contained 20% (0.9 g per 100 g of total protein) of lysozyme measured by using HPLC. In addition, the SDS−PAGE analysis revealed the presence of trace amounts of ovotransferrin and ovalbumin, but the authors did not quantify the amounts of these proteins. In the present study, liquid egg white was found to contain about 6.8 g of crude ovomucin per 100 g of total protein and the purity factor for our preparation was calculated to be 71% (**Table 2**) as compared to 80% reported by Guérin and Brulé (*18*). However, their purity factor was somewhat overestimated because they did not quantify the coprecipitated ovotransferrin and ovalbumin. Nevertheless, using the obtained purity factors it could be calculated that our crude ovomucin preparation contained 5.0 g of ovomucin per 100 g of total protein, while Guérin and Brulé (*18*) obtained 3.8 g of ovomucin per 100 g of total protein. Finally, the filtration byproduct had a crude ovomucin content of about 820 mg per 100 g of egg albumen. The actual ovomucin content was calculated to be about 520 mg in 100 g of egg albumen. Unfortunately, to our knowledge, there are no previously reported data on the ovomucin content of the filtration byproduct fraction.

Thus, to summarize, the present study revealed rather large differences between the ovomucin contents of the different egg albumen fractions. Both the liquid egg albumen and the filtration byproduct fraction contained almost twice the amount of ovomucin in whole egg albumen. The high ovomucin content of the liquid egg albumen fraction as compared to the whole egg albumen fraction is quite surprising, because basically there should not be any apparent difference between these two fractions. However, in this study, the eggs for the whole egg white fraction studies were obtained from the local hen house, whereas the liquid egg white was obtained from an egg processing plant. Thus, there are evidently some differences between these two fractions due to different hen hybrids, hen ages, etc. Moreover, in the case of the whole egg albumen studies, we removed all of the chalazae cords from our samples. In contrast, even though most of the chalazae cords are filtered off in the egg processing plant prior to the pasteurization of liquid egg, it is very likely that some of them will pass through the sieve and end up in the liquid egg white fraction.

An interesting factor in comparing different ovomucin fractions in terms of their biological activity is, of course, their carbohydrate content. The carbohydrate contents for crude ovomucins measured in this study are presented (in boldface) in **Table 3**. The table also shows the previously published carbohydrate contents of whole and thick egg white ovomucins. We found no reported data for liquid egg white or filtration byproduct ovomucins. As can be seen in **Table 3**, the results of previous studies vary considerably, especially with regard to the total carbohydrate contents of thick egg white ovomucins, where the results range from 19.5 to 34.7%. Evidently, the different degrees of purity between the ovomucin precipitates are again the major reason for these variations. The total carbohydrate contents obtained in this study are quite well in accordance with the results of earlier studies. However, we are aware that the sialic acid content found in our ovomucin preparations is quite low as compared to previously reported values in the case of thick egg white ovomucin. The reason for this, however, is unclear.

As shown in **Table 3**, the total carbohydrate content in this study varied from 20.3% of the filtration byproduct crude ovomucin to 23.6% of whole egg white crude ovomucin. The variation among the different crude ovomucins, therefore, was quite small. On the other hand, there was more variation in the crude ovomucin contents of the different albumen fractions, and the amount of total carbohydrate in milligrams varied concurrently. Naturally, when egg albumen ovomucin is used as an ingredient for functional foods, the total carbohydrate content obtained in milligrams is virtually more important than the percentage-based carbohydrate content. We also calculated these values by using the amounts of different egg albumen crude ovomucins obtained from 100 g of egg albumen. These values are also given in **Table 3**. The variance among the different egg albumen fractions is much greater when the amounts of

Table 4. Shares (% of Ovomucin) and Amounts (mg/100 g of Albumen) of Ovomucin Subunits

	β-ovomucin		α2-ovomucin		α1-ovomucin	
egg white fraction	% of ovomucin	mg/100 g of albumen	% of ovomucin	mg/100 g of albumen	% of ovomucin	mg/100 g of albumen
whole egg white	9	25	83	230	8	20
thick egg white	11	40	86	290	3	10
liquid egg white	16	80	73	365	11	55
filtration byproduct	25	130	61	315	14	75

carbohydrate obtained from the different crude ovomucins are compared in this way. About 100 mg of carbohydrates was obtained from 100 g of whole white albumen, whereas the filtration byproduct contained about 165 mg.

The third item we wanted to examine in this study was the β-ovomucin content in the different egg albumen ovomucins, since it has been reported that β-ovomucin or some of its fragments are essential for some bioactive properties of ovomucin (*7*, *10*). We used the peak areas of the elution profiles of the different ovomucin samples obtained by GFC (**Figure 1**) to approximate the amounts of different ovomucin subunits. The gel filtration system measured the absorbance at 280 nm for protein, which may have led to an underestimation of the β-ovomucin content, since β-ovomucin is a carbohydrate-rich subunit. Nevertheless, this system allowed us to compare the different ovomucin fractions studied here. **Table 4** shows the amounts of different subunits in the ovomucin samples. Again, there were great variations between the different albumen fractions. Whole egg albumen contained about 25 mg of β-ovomucin in 100 g of albumen, while thick albumen, liquid egg albumen, and the filtration byproduct contained about 1.5, 3, and 5 times more β-ovomucin, respectively. As noted above, many studies have shown that thick albumen contains most of the β-ovomucin present in egg albumen. In the case of the filtration byproduct, the high β-ovomucin content might be explained by chalazae cords and possibly some yolk membranes, restrained into this albumen fraction during the filtration procedure. However, the reasons for the much higher β-ovomucin content in liquid egg albumen as compared to whole egg albumen are very likely to be the same as those used above to explain the large difference in ovomucin content between the whole egg and liquid egg white fractions.

CONCLUSIONS

In conclusion, both the filtration byproduct and the liquid egg mass appear to be potential alternatives to whole egg albumen and thick egg albumen as sources of ovomucin. Both of those fractions contained about 2 and 1.5 times the amount of ovomucin as compared to whole and thick egg white, respectively. They also contained more β-ovomucin, the carbohydrate-rich subunit of ovomucin. The potential of the filtration waste should be particularly good news to the egg industry, because so far it has been used as a more or less low-priced feed. It should be noted that although ovomucin is easily fractionated from egg white by using IEP, the ovomucin preparation thus obtained is highly insoluble in water or in conventional buffer solutions, which limits its straightforward use as a food ingredient. However, enzymatic hydrolysis has been suggested as an alternative way of enhancing the solubility of ovomucin (*40*, *46*−*49*). Thus, some interesting questions remain, namely: Are there differences in biological activities among different ovomucin fractions, and furthermore, how does the enzymatic hydrolysis affect ovomucin's biological activities? Further studies are now underway in our laboratory to elucidate these questions.

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