Isolation of Immunoglobulin from Egg Yolk by Anionic Polysaccharides

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Isolation conditions of immunoglobulin in egg yolk (IgY) were optimized by the addition of various levels of Na-alginate (Alg), λ-carrageenan (λ-Cg), Na-carboxymethyl cellulose (CMC), and pectin (PC) to 6-fold diluted yolk. The mixtures were then reacted at pH 4.0−6.0 for 30 min. The optimal isolation conditions of IgY for Alg, λ-Cg, and CMC were at the 0.1% level and at pH 5.0, while those for PC were at the 0.15% level and at the same pH. The remaining lipid and remaining protein in the supernatants thus obtained was 0.5−3.8% and 10−17%, respectively, and more than 90% of lipoproteins were precipitated. The IgY recovery was determined to be 33−74% by means of single radial immunodiffusion method when IgY was isolated under the optimal conditions. PC showed the best recovery of IgY, while λ-Cg provided the least. The interactions between polysaccharides and lipoproteins were mainly ionic bonds, hydrophobic interactions, and hydrogen bonds as determined by the addition (0−2.0 M) of NaSCN or urea to the polysaccharide−lipoprotein precipitates.

Keywords: *Anionic polysaccharide; lipoprotein; interaction; isolation; immunoglobulin in yolk (IgY)*

INTRODUCTION

Immunoglobulins have been proven to have immunoprophylatic or therapeutic potential and can be used as immunological supplements in infant formulas and other foods (Goldman, 1989; Facon et al., 1993). Specific antibodies from the colostrum of immunized cows have been found to be effective in the prevention of *Escherichia coli* (Mietens et al., 1979; Tacket et al., 1988), rotavirus (Ebina et al., 1985; Hilpert et al., 1987), cryptosporidum (Tzipori et al., 1986), and *Shegella flexneri* (Tacket et al., 1992). However, colostral milk is not available in large amounts, which makes it unreliable as a source of immunoglobulins.

The high immunoglobulin content (approximately 10 mg/mL) in egg yolks (Otake et al., 1991), the ease of its collection, as well as the large quantities of chicken eggs available make IgY a potential source for immunological supplementation of foods. Many researchers (Bartz et al., 1980; Hamada et al., 1991; Hatta et al., 1993; Chang et al., 1999) have already developed chicken eggs with specific antibodies from immunized hens.

Due to increasing demands for food use, IgY has become increasingly important in food processing, and therefore, there is a need for developing efficient and simple techniques for IgY production. Some methods, such as gel permeation chromatography (Burley and Vadehra, 1979), addition of poly(ethylene glycol) (Polson, 1985; Akita and Nakai, 1993) and dextran sulfate (Jensenius, 1981; Akita and Nakai, 1993), and the combination of ethanol precipitation with DEAE-cellulose ion-exchange chromatography (Bade and Stegemannn, 1984) have been investigated. However, most of these methods suffer from major drawbacks, i.e., low recovery rates of IgY or the complexity of the procedures, and they appear impractical for routine use in food items. Thus, an efficient, economic, and rapid method for separation of IgY from yolk is needed.

Recently, λ-carrageenan (Hatta et al., 1990), alginate (Hatta et al., 1988), and hydroxypropylmethylcellulose phthalate (Ikemori et al., 1992) have been used to recover IgY from adequately diluted yolks in a single-step process, and those polysaccharides exhibit satisfactory yields of IgY. Interactions among myofibrillar proteins, polysaccharides (such as alginate and pectin), and calcium were reported to be electrostatic forces, hydrophobic interaction, and hydrogen bonds (Bernal et al., 1987), while only electrostatic forces and hydrogen bonds were observed between myofibrillar proteins and alginate (Ustunol, 1992). However, reactions between yolk lipoproteins and polysaccharides have been rarely reported.

In this study, IgY was recovered from diluted yolk by the addition of various polysaccharides such as alginate, Na-carboxymethyl cellulose, pectin, and λ-carrageenan, and the optimal conditions for each polysaccharide were investigated. Furthermore, in order to understand the mechanisms of polysaccharide−lipoprotein interactions, NaSCN and urea were mixed with polysaccharide−lipoproteins precipitates and their changes in absorbance at 660 nm were recorded.

MATERIALS AND METHODS

Fresh eggs were purchased from a local supermarket. The approximate composition of chicken egg yolks used, determined according to AOAC (1984), was moisture 49.42 ± 0.99%,

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protein 15.90 ± 0.46%, lipid 33.49 ± 0.91%, and ash 1.51 ± 0.16%.

Alginate (Alg), λ-carrageenan (λ-Cg), Na-carboxymethyl cellulose (CMC), pectin (PC) (catalog No. 37251-70-0, DE = 67), NaSCN, and urea were all purchased from Sigma Co., St. Louis, MO.

Preparation of Crude IgY Solution. Yolk, separated from fresh chicken albumen, was diluted 2-fold with equal volumes of 0.01 N acetate buffer (pH 4.0−6.0) and was then mixed well with 2 volumes of one of 0.05−0.15% Alg/-, 0.01−0.15% λ-Cg/-, 0.05−0.15% CMC/-, or 0.10−0.20% PC/0.01 N acetate buffer (pH 4.0−6.0). After being incubated at ambient temperature for 30 min, centrifugation (10000*g*, 15 min, 4 °C) was conducted to remove the precipitates. The relative amounts of IgY, residual lipid content, and residual protein content, determined by the methods described below, in the supernatants (crude IgY) were calculated to optimize the separation conditions for each polysaccharide used in the present study.

Determination of Protein. Protein in the collected polysaccharide−lipoprotein precipitates was determined by the micro Kjeldahl method (AOAC, 1984). Protein content in the supernatants of polysaccharide−yolk mixtures was determined by the method described by Bradford (1976). Microtiter plate protocol of Bio-Rad protein assay was conducted, and chicken serum immunoglobulin G (IgG) (Sigma, St. Louis, MO) was used as the standard solution (0.1−0.6 mg of IgG/mL) to prepare the standard curve (McCannel and Nakai, 1990). Triplicate samples were each analyzed twice for protein content.

$$\text{remaining protein (\%)} = \text{protein (mg) in the supernatant/ protein (mg) in the 2-fold diluted yolk} \times 100\% \quad (1)$$

Determination of Lipid. Lipid was extracted from diluted yolks using a solvent of chloroform−ethanol mixture (chloroform−ethanol = 3:1 v/v) as described by Hatta et al. (1990). Triplicate samples were each analyzed twice for lipid content.

$$\text{remaining lipid (\%)} = \text{lipid (mg) in the supernatant/ lipid (mg) in the 2-fold diluted yolk} \times 100\% \quad (2)$$

Determination of Phosphorus. Phosphorus content in the 2-fold diluted yolk and polysaccharide−lipoprotein precipitates was determined with a standard method (Bartlett, 1958). Adequate amounts of samples were treated with perchloric acid, ammonium phosphomolybdate, and amidol, and then the absorbance at 830 nm was recorded. A standard curve (phosphorus content 0.5−10 μg/mL) for the quantification of phosphorus was prepared with NaH_2PO_4. Triplicate samples were each analyzed twice for phosphorus content.

Single Radial Immunodiffusion (SRID). The relative amount of IgY in the 2-fold diluted yolk solution and crude IgY was determined with SRID plates containing rabbit anti-chicken IgG (Sigma, St. Louis, MO) (Hudsan and Hay, 1989). SRID plates were incubated at room temperature (27 ± 2 °C) in a moist chamber until there was no further increase in precipitin ring diameter (about 48 h). Chicken serum IgG was used as the standard solution (0.1−2.0 mg IgG/mL) to prepare the standard curve, which was plotted with the square of the diameter of precipitin ring versus IgY concentration. The IgY content in fresh yolk was determined to be 1.57 ± 0.05%. Purity (%) of IgY in supernatants of polysaccharide-yolk mixtures = IgY (mg) in the supernatant/protein (mg) in the corresponding supernatant. Triplicate samples were each analyzed twice for IgY content.

Determination of Lipoprotein−Polysaccharide Interaction. To elucidate the importance of ionic bonds and hydrogen bonds on the formation of polysaccharide−lipoprotein precipitates, influences of NaSCN, with charge neutralization effect, and urea on the changes in turbidity of precipitates were studied (Bernal et al., 1987). In this study, a certain amount of precipitate collected from each polysaccharide−lipoprotein mixture was added to adequate amounts of NaSCN or urea to prepare solutions with 0.01−2.0 M NaSCN or urea.

Table 1. Remaining Lipid[a] (%) and Remaining Protein[b] (%) in the Supernatants of Polysaccharide−Yolk Mixtures[c]

	concn (%)	pH	remaining lipid (%)	remaining protein (%)
(A) Na-alginate	0.05	4.0	71.91 ±1.69	55.68 ± 1.30
	0.10	4.0	64.78 ± 5.11	44.99 ± 3.71
	0.15	4.0	80.59 ± 1.07	50.17 ± 0.67
	0.05	5.0	7.53 ± 0.76	18.94 ± 1.92
	0.10	5.0	1.64 ± 0.05	11.60 ± 0.36
	0.15	5.0	1.78 ± 0.03	12.54 ± 0.20
	0.05	6.0	7.45 ± 0.02	18.75 ± 0.55
	0.10	6.0	7.00 ± 0.13	17.62 ± 0.34
(B) λ-carrageenan	0.01	4.0	83.02 ± 1.51	70.92 ± 0.51
	0.05	4.0	75.75 ± 0.31	64.66 ± 0.26
	0.10	4.0	60.56 ± 0.26	50.48 ± 0.22
	0.15	4.0	56.65 ± 1.67	35.22 ± 0.94
	0.01	5.0	2.82 ± 0.24	18.07 ± 0.34
	0.05	5.0	1.87 ± 0.04	15.02 ± 0.31
	0.10	5.0	0.50 ± 0.01	10.15 ± 0.27
	0.15	5.0	0.41 ± 0.02	8.32 ± 0.19
	0.01	6.0	52.00 ± 4.68	35.88 ± 3.23
	0.05	6.0	9.19 ± 0.28	23.51 ± 0.73
	0.10	6.0	7.94 ± 0.56	19.39 ± 1.37
(C) Na-carboxymethyl cellulose	0.05	4.0	89.58 ± 5.23	88.45 ± 1.68
	0.10	4.0	87.01 ± 0.76	61.56 ± 4.55
	0.15	4.0	9.08 ± 0.68	34.21 ± 1.52
	0.05	5.0	8.07 ± 0.13	18.33 ± 0.18
	0.10	5.0	3.55 ± 0.33	16.47 ± 1.59
	0.15	5.0	5.11 ± 0.30	20.35 ± 0.73
	0.05	6.0	7.23 ± 0.05	23.76 ± 0.37
	0.10	6.0	5.90 ± 0.20	22.23 ± 0.16
(D) pectin	0.10	4.0	74.05 ± 0.82	65.61 ± 0.73
	0.15	4.0	49.74 ± 1.59	44.44 ± 0.89
	0.20	4.0	17.54 ± 1.13	22.83 ± 0.30
	0.10	5.0	4.04 ± 0.03	17.88 ± 0.15
	0.15	5.0	3.83 ± 0.05	16.97 ± 0.19
	0.20	5.0	4.65 ± 0.31	19.53 ± 0.18
	0.10	6.0	48.89 ± 1.02	32.75 ± 0.68
	0.15	6.0	45.42 ± 0.59	30.43 ± 0.40
	0.20	6.0	29.57 ± 1.23	22.26 ± 0.10

[a] Lipid (mg) in the supernatant/total lipid (mg) in the starting yolk solution ×100%. [b] Protein (mg) in the supernatant/ total protein (mg) in the starting yolk solution ×100%. [c] Mixtures of 6-fold diluted egg yolk and various levels of polysaccharides at different pH values were centrifuged (1000*g*, 15 min) after incubation for 30 min to obtain the supernatants. Average ± deviation of three replicates.

After incubation at ambient temperature for 30 min, the mixtures were then centrifuged (5000*g*, 5 min) and the absorbance of supernatants at 600 nm was recorded. Triplicate samples of each precipitate−NaSCN or precipitate−urea solution were analyzed in duplicate for absorbance.

RESULTS AND DISCUSSION

Optimization of the Isolation of IgY. Alg (0.05, 0.10 and 0.15%) was added to the diluted yolk at pH 4.0, 5.0, and 6.0, and the remaining lipoprotein in the supernatants was investigated. It was found that the remaining lipid was 65−81% and the remaining protein was 45−56% when reactions occurred at pH 4.0 (Table 1A). However, when the pH of the reaction mixtures was increased to 5.0, the remaining lipid was reduced to1.6−7.5% and the remaining protein was 11−19%. A further increase in the pH of the Alg-yolk reaction mixtures to 6.0 brought the remaining lipid to 6.0−7.5% and the remaining protein to 14−19%. Imeson et al. (1977) have reported that protein and polysaccharide could form complexes through electrostatic forces when protein is at a pH lower than the pI and in low ionic strength. Therefore, the formation of complexes could be hindered

by the ionic strength and pH of the protein−polysaccharide mixture (Chang et al., 1987). The pH of the solution influences the charge of protein molecules, as well as the dissociation of carboxyl groups in Alg, CMC, and PC or of the sulfate groups in λ-Cg. Thus, the changes in pH apparently affect the extent of interactions between polysaccharides and proteins and the degree of precipitation of polysaccharide−lipoprotein complexes (Samant et al., 1993; Gurov et al., 1983). Tolstoguzov (1991) indicated that the pH of the reaction mixtures affected the formation of complexes. The optimal condition for Alg to remove lipoproteins from 6-fold diluted yolk was found to be Alg 0.1% at pH 5.0.

Table 1B presents the use of λ-Cg (0.01, 0.05, 0.10, 0.15%, pH 4.0, 5.0, 6.0) for removing lipoproteins from diluted yolk. It was found that the remaining lipid was 57−83% and remaining protein was 35−71% when λ-Cg was reacted with yolk solution at pH 4.0. Increasing the pH of the λ-Cg-yolk solution to 5.0 also reduced the remaining lipid to 0.4−2.8% and the remaining protein to 8−18%, displaying a trend similar to the use of Alg. At pH 6.0, the remaining lipid was determined to be 7−52% and the remaining protein was 19−36%, much higher than the values at pH 5.0. Samant et al. (1993) indicated that polysaccharides containing sulfate groups react with globulin to form precipitates at pH levels higher than the isoelectric point of proteins. The optimal condition for λ-Cg to remove the lipoproteins from 6-fold diluted yolk was found to be λ-Cg 0.1% at pH 5.0. Hatta et al. (1990) have reported that the use of 0.1% λ-Cg displayed results of remaining lipid 0.4% and remaining protein 20.2%, similar to the results in the present study.

CMC (0.05, 0.10, 0.15%) with carboxyl groups also reacted with 6-fold diluted yolk at various pHs (4.0, 5.0, 6.0), and the results were as shown in Table 1C. At pH 4.0, the remaining lipid was 9−90% and the remaining protein was 34−88%. Similar to the previous results obtained with Alg and λ-Cg at pH 5.0, the remaining lipid (3−8%) and the remaining protein (16−20%) were the least when CMC and the yolk solution was reacted at pH 5.0. The optimal condition for CMC to remove lipoproteins from 6-fold diluted yolk was concluded to be CMC 0.1% at pH 5.0.

Pectin (0.10, 0.15, and 0.20%) was reacted with 6-fold diluted yolk at pH 4.0, 5.0, and 6.0, and the results are shown in Table 1D. The remaining lipid and the remaining protein in the supernatant of the PC−yolk solution, which varied with the pH of the reaction mixture, reduced initially at pH 5.0 but increased subsequently when the pH was increased to 6.0. The optimal condition for PC to remove lipoproteins from 6-fold diluted yolk was determined to be PC 0.15% at pH 5.0.

The difference in the amount of remaining protein could be due to the variations in conformational structure of polysaccharide used and in the dissociation constants of carboxyl groups in Alg, PC, and CMC and sulfate group in λ-Cg. In recovering lysozyme from egg albumen, Yang et al. (1998) indicated that protein−polysaccharide interactions were not only caused by electrostatic forces, but also by the type of charged groups in the polysaccharide.

The IgY contents in the crude IgY solutions prepared with four polysaccharides under the respective optimal conditions were determined by SRID, and the purity and the immunoactivity recovery of IgY are shown in Table 2. PC appeared to be the most effective in recovering immunoactive IgY (74%), followed by CMC (60%), Alg (39%), and λ-Cg (33%), and also most effective in purifying IgY (purity 21%). Hatta et al. (1990) reported that the use of 0.1% λ-Cg of food-additive grade exhibited a 86% yield of IgY from the doubly diluted yolk in the nature pH value. The large difference in recovery of IgY could be due to the variation of λ-Cg in sources and purity, as well as in isolation procedure.

Table 2. Immunoactivity Recovery[a] and Purity[b] of IgY in Supernatants[c] of Polysaccharide−Yolk Mixtures

polysaccharide	level (%)	purity (%)	immunoactivity recovery (%)
Na-alginate	0.10	15.05 ± 0.10	38.96 ± 1.66
λ-carrageenan	0.10	19.30 ± 0.31	33.25 ± 0.57
Na-carboxymethyl cellulose	0.10	20.19 ± 0.12	60.12 ± 0.13
pectin	0.15	20.87 ± 0.10	73.92 ± 3.24
control[d]		9.89 ± 0.22	100

[a] IgY (mg) in the supernatant/IgY (mg) in the 2-fold diluted yolk × 100%. [b] IgY (mg) in the supernatant/protein (mg) in the supernatant × 100%. [c] Polysaccharide−yolk mixture was centrifuged (1000*g*, 15 min) after incubation for 30 min. [d] 6-fold diluted fresh yolk Average ± deviation of three replicates.

Table 3. Percentage of Phosphorus in Polysaccharide−Yolk Precipitates Relative to Total Phosphorus in the Starting Yolk Solution

polysaccharide	concn (%)	percentage[a] (%)
Na-alginate	0.10	95.81 ± 2.35
λ-carrageenan	0.10	99.74 ± 7.24
Na-carboxymethyl cellulose	0.10	91.92 ± 1.85
pectin	0.15	90.89 ± 1.66

[a] Phosphorus (mg) in the precipitates/total phosphorus (mg) in the 2-fold diluted yolk × 100 (%). Average ± deviation of three replicates

Analysis of Polysaccharide−Lipoprotein Precipitates. The phosphorus contents of the precipitates, collected from various polysaccharide-yolk mixtures prepared under the respective optimal conditions, were also determined. It was found that the phosphorus in the precipitates from every polysaccharide−yolk mixture were more than 90% of the total phosphorus in the starting diluted yolk (Table 3). The reaction of anionic polysaccharides used in the present study with lipoproteins in diluted yolk appeared to be remarkable, and the lipoproteins were almost entirely precipitated by those polysaccharides (Table 1). The addition of 0.7% κ-carrageenan was also effective in recovering 78−81% lysozyme from fresh hen egg whites (Yang et al., 1998).

Interactions between Anionic Polysaccharides and Lipoproteins. Various concentrations of NaSCN in polysaccharide−yolk mixtures were prepared to investigate the possible effects of ionic bonds and hydrophobic interactions on the polysaccharide−lipoprotein interactions. As can be seen in Figure 1, turbidity (absorbance 600 nm) of Alg and PC precipitates increased sharply to about 2.3, relatively higher than those of CMC (1.3) and λ-Cg (1.0), when the NaSCN level was adjusted to 0.2 M. NaSCN solution destroys the ionic bonds of complexes at levels under 1.0 M, while it weakens the hydrophobic interactions of complexes at NaSCN levels higher than 1.0 M (Bernal et al., 1987). Thus, the increase in the turbidity of precipitates (Alg and PC) at the lower level (0.2 M) of NaSCN indicated the easier destruction of polysaccharide−protein interactions. Rapid decline of turbidity (1.1) of PC precipitates was recorded at 0.5 M NaSCN solution, while the

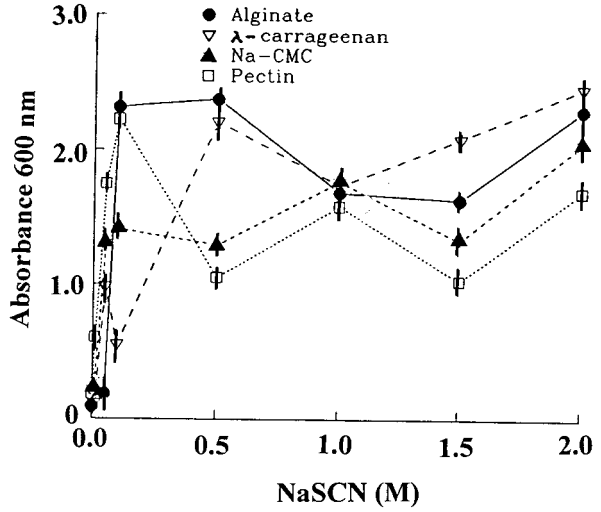

Figure 1. Changes in turbidity of anionic polysaccharide−lipoprotein complexes under various NaSCN concentrations. Each value is the average of three determinations.

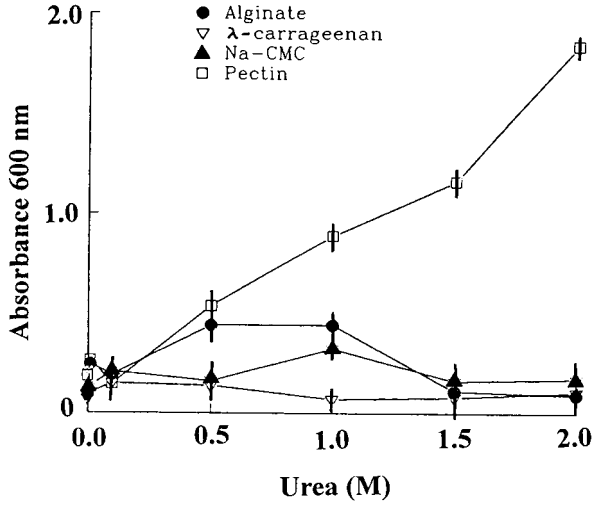

Figure 2. Changes in turbidity of anionic polysaccharide−lipoprotein complexes under various urea concentrations. Each value is the average of three determinations.

turbidity (2.4) of Alg precipitates remained high. However, turbidity of λ-Cg precipitates rapidly increased to 2.2 at the NaSCN level of 0.5 M, indicating the destruction of ionic bonds at the NaSCN level between 0.2 and 0.5 M. It is noteworthy that a further increase of the NaSCN level in the precipitates to 1.0 M reduced the turbidities of Alg and λ-Cg precipitates to about 1.8, close to those (1.7−1.8) of CMC and PC precipitates at the same NaSCN level. Such results present almost the same degrees of destruction of ionic bonds in each polysaccharide−protein precipitates in the 1.0 M NaSCN. From the trends of turbidity changes of precipitates at NaSCN levels lower than 1.0 M, it was found that the decreasing extent of the importance of ionic bonds on the formation of polysaccharide−protein precipitates was Alg > PC > λ-Cg > CMC. A higher level (2.0 M) of NaSCN in all of the precipitates increased the turbidity and, in other words, made the polysaccharide−protein precipitates unstable. The decreasing extent of the importance of hydrophobic interactions on the formation of polysaccharide−protein precipitates was estimated to be λ-Cg > Alg > CMC > PC.

Investigations on the effect of urea on the stability of polysaccharide−lipoprotein precipitates were conducted to determine the importance of hydrogen bonds on the complex formations. As shown in Figure 2, only slight increases in the absorbance (0.2−0.4) of Alg and CMC precipitates at 600 nm in 0.5−1.0 M urea were observed, while a sharp increase in the absorbance (1.9) of PC precipitates in 0−2.0 M urea was detected. The λ-Cg−lipoprotein complexes appeared not to be influenced in the presence of urea (0−2.0 M). Thus, hydrogen bonds were found to be most influential to the formation of PC-lipoprotein complexes, followed by the formation of Alg precipitates, CMC precipitates, and λ-Cg precipitates.

CONCLUSION

Anionic polysaccharides were added to diluted yolk to isolate IgY by removing lipoproteins, and the experimental conditions for each polysaccharide were optimized. It was found that the pH value of the polysaccharide−yolk mixture was influential to the quantity of polysaccharide−lipoprotein precipitates and to the immunoactivity recovery of IgY. Among the polysaccharides tested in the present study, the use of 0.15% PC exhibited the best immunoactivity recovery of IgY when PC was reacted with 6-fold diluted yolk solution at pH 5.0. The interactions of polysaccharide and lipoproteins were mainly ionic bonds, hydrophobic interactions, and hydrogen bonds. However, the extent of each force on the formation of polysaccharide−lipoprotein complexes was dependent on the source of polysaccharide used. Rapid removal of lipoproteins from yolk could be beneficial for food industries to prepare crude IgY as an immunological supplement for infant formulas and other foods, especially using eggs from hens specifically immunized.

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