ELECTROPHORETIC AND IMMUNOCHEMICAL PROPERTIES OF THE 12S RAPESEED PROTEIN

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ABSTRACT

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The 12S rapeseed protein as prepared by gel filtration of an alkaline extract of commercial meal was subjected to polyacrylamide disc gel electrophoresis and immunoelectrophoresis, both by conventional means and in the presence of sodium dodecyl sulfate (SDS). The 12S isolate was separated into a major component and a slower moving minor component by disc gel electrophoresis, while SDS gel electrophoresis resulted in the separation of subunits with apparent molecular weights of approximately 42,000, 37,600, 30,100, 17,400, and 12,200 daltons. Electrophoretic patterns of nonreduced and reduced samples indicated the presence of intermolecular disulfide bonds, although the cystine content was low. The isolate contained a high molecular weight component, but immunoelectrophoretic analysis resulted in the formation of one homogeneous pair of precipitin arcs. This would suggest that the 12S protein self-associates to form aggregates of higher molecular weight (dimers). Schiff staining of SDS gels indicated that the fragment with the lowest molecular weight contained most of the carbohydrate. This fragment was immunoresponsive in the presence of SDS, whereas the other subunits were not. One explanation for this phenomenon could be that the low molecular weight glycopeptide is located on the surface of the aggregate.

In the past decade, rapeseed proteins have received considerable attention because of their potential uses in human nutrition. The rapeseed 12S protein with a molecular weight of 130,000 daltons (1), which was first recovered from crushed seed by a series of chromatographic procedures (2), perhaps has been the most intensively studied.

Recent work has indicated, however, that composition of the 12S protein recovered from commercial meal (*Brassica campestris* L. var. Span) may differ from the composition of that recovered from the seeds of other species (3). Characterization of the 12S protein from rapeseed meal is particularly relevant, as the defatted meal, a byproduct of the oil extraction procedure, is the most economic rapeseed protein source. In spite of the differences, all of the 12S preparations to date have contained small but significant levels of 17S protein as well as some low molecular weight material (1,2,4). Bhatty et al (2) reported that the 12S protein from seed readily dissociates at pH values below 3.5 and in urea. More recently, MacKenzie (5) suggested that the 12S protein recovered from oriental mustard (*B. juncea*) self-associates to form 15S and 18S aggregates, which readily dissociate along with the 12S material in urea solutions. Because of the difficulties encountered in determining the homogeneity of preparations suspected of being self-associating, serologic methods were used to characterize the isolate prepared from rapeseed meal.

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MATERIALS AND METHODS

The 12S protein was isolated from commercial defatted rapeseed meal (*B. campestris* L. var. Span) as previously described (1).

Electrophoresis

Polyacrylamide gel electrophoresis was performed on the lyophilized protein by the method of Davis (6), using 7% acrylamide gels. All gels were run in a Pharmacia gel electrophoresis apparatus, Model GE-4, at 5 mA per tube. Gels were stained with Coomassie blue (7) for a period of 8–12 hr and destained in a Pharmacia gel destainer, Model GD-4, with a mixture of 5% methanol and 7% acetic acid. Gels were scanned in a Transidyne TG 2980 automatic scanning densitometer at 550 nm. Electrophoresis was also performed in the presence of sodium dodecyl sulfate (SDS) using a buffer system that Neville (8) described. The SDS gel system, which used a sulfate-borate discontinuity, stacked at pH 8.64, and ran at pH 9.5, was modified as follows: Lower gels (running gels) were prepared from 0.1% N,N′-methylenebisacrylamide. Upper gels (stacking gels) were prepared from 3.1% acrylamide containing 0.2% N,N′-methylenebisacrylamide, contained 20% sucrose and 0.1% SDS, and were photopolymerized with 5×10^{-5}% riboflavin as a catalyst. Samples and molecular weight markers were dissolved in stacking buffer containing 8*M* urea, 1% SDS, and 0.14*M* dithiothreitol and heated in a boiling-water bath for 5 min. The gels were loaded with between 10 and 50 μg of protein.

Electrophoresis was performed at 2 mA per tube at 15° C. Protein bands were detected with Coomassie blue and glycoprotein components detected by a modification of the procedure that Glossmann and Neville (9) described. They reported that for several glycoproteins (eg, ovomucoid and α_1-glycoprotein), the Coomassie-stained bands represent minor contaminants, whereas the periodic acid-Schiff-positive (PAS-reactive) bands represented the true molecular subunits. Many carbohydrate-rich glycoproteins stained only faintly with Coomassie blue. To avoid any possibility of artifactual staining with Coomassie blue, the glycoprotein molecular weight markers were located by both Coomassie blue and Schiff reagent. The PAS procedure was accomplished by washing the gels overnight (two changes) in the GD-4 destainer with 40% methanol in 7% acetic acid to remove bound and unbound SDS. All gels except controls were oxidized for 1 hr in 1% periodic acid in 7% acetic acid in the dark and transferred into 0.5% sodium arsenite in 5% acetic acid (10) for 1 hr. Three 20-min changes of 0.1% sodium arsenite in 5% acetic acid were followed by a 20-min wash in 5% acetic acid. All gels, including controls, were stained with Schiff reagent overnight at 4° C in the dark. The unreacted Schiff reagent was removed with several rinses of 1% sodium metabisulfite in 0.1*N* HCl. Apparent molecular weights were calculated for the 12S subunits by the method of Weber and Osborn (7) using a standard curve prepared from the relative mobilities of bovine thyroglobulin (335,000 mol wt), conalbumin (86,180 mol wt), ovalbumin (43,500 mol wt), and bovine pancreatic RNase (12,640 mol wt).

Immunoelectrophoresis

Disc immunoelectrophoresis (11,12) was undertaken in this study to establish the immunologic identity of the 12S rapeseed protein.

Preparation of the Antisera. Two species were selected for production of precipitating antibody. In the first experiment, 18 white mice were injected with a crude extract of whole rapeseed protein (WRE). WRE was prepared by the overnight extraction of commercial rapeseed meal with 0.1*M* borate buffer, pH 9.2. The filtered extract was stirred at 4° C and the proteins precipitated by addition of granular ammonium sulfate (70%, w/v) over a period of 30 min. The precipitated protein was dialyzed against cold running water for 72 hr and then 0.01*M* sodium phosphate buffer, pH 7.6, for 48 hr at 4° C. Protein concentration was determined by micro Kjeldahl analysis (N × 6.25). A 1.3% protein suspension was mixed 1:1 with Freund's complete adjuvant and 0.1 ml of the mixture administered intraperitoneally by a procedure similar to that used by Catsimpoolas and Meyer (11). Two subsequent injections of the same size were given at one-week intervals. After a rest period of four weeks, the animals were given a booster injection of 0.1 ml and bled by cardiac puncture two weeks later. The blood was pooled and allowed to clot at room temperature for approximately 1 hr and then refrigerated overnight. The clots were carefully cut and separated from the serum by centrifugation.

In the second experiment, four single-comb white leghorn roosters were used. Two were injected with a 1:1 mixture of Freund's complete adjuvant and WRE (0.90% protein) prepared as before and two with an aqueous sample of 12S protein (0.54% protein) 1:1 in Freund's complete adjuvant. The birds were given 1 ml of antigen intraperitoneally in the first week, 2 ml in the second, and 3 ml in the third. The birds were given 5-ml booster injections after a 30-day rest period and again one week later. After two more weeks, the birds were bled by cardiac puncture, and the serum was collected.

Electrophoresis and Diffusion. Samples (130–400 μg) of the 12S protein were electrophoresed by Davis' (6) method on 4% acrylamide gels. The unstained gels were then cast in petri dishes filled with 1% Ionagar No. 2 (Oxoid) in pH 8.6 sodium diethylbarbiturate-sodium acetate buffer, ionic strength 0.05. The agar contained 0.02% sodium azide as a preservative. After solidification, trenches a few millimeters deep were cut parallel to the gel columns but separated at a distance of approximately 1 cm and filled carefully with the mouse anti-WRE antiserum. The reactants were allowed to diffuse at room temperature in a glass desiccator containing a small amount of water. Gels containing the same antigen

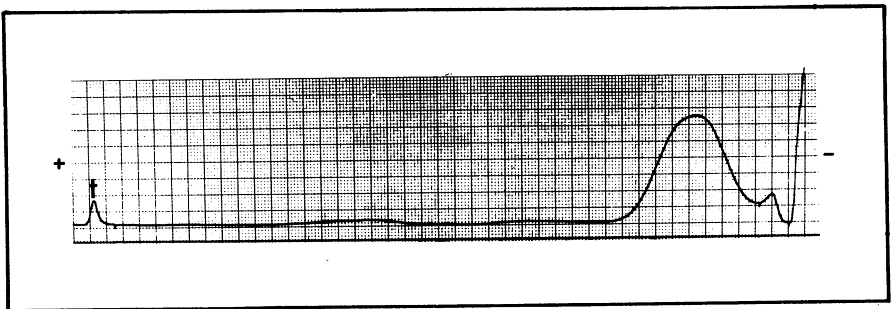

Fig. 1. Densitometric scan of disc gel of 12S glycoprotein isolated from commercial rapeseed meal. t, position of bromphenol blue tracking dye.

loads were electrophoresed along with the above gels and stained with Amido black 10B to locate the protein band. Antigen (12S protein) was also diffused against anti-WRE by the Ouchterlony (13) method using the same agar.

Similar procedures were performed with the rooster anti-WRE and the anti-12S antisera. WRE was first dialyzed against electrode buffer and immunoelectrophoresed against rooster anti-12S antiserum. Lyophilized 12S protein was dissolved directly in electrode buffer and immunoelectrophoresed against rooster anti-WRE serum. Protein loads ranged from 50 to 200 μg for the 12S isolate and 20 to 80 μg for the WRE. Control gels were stained with Coomassie blue, which was found to be more sensitive and yielded superior resolution compared with Amido black.

Immune SDS gels were run as described above, each gel being loaded with 10–400 μg of the dissociated 12S isolate. After electrophoresing for approximately 1.5 hr at 2 mA per gel, the positions of the bromphenol blue tracking dye were marked with drafting ink and the gels embedded in Ionagar No. 2. Trenches were cut and filled with rooster anti-12S antiserum and the reactants allowed to diffuse. The white precipitin bands that formed were stained with Amido black and the results recorded photographically.

RESULTS AND DISCUSSION

The proteins of the 12S aggregate are oligomeric, dissociating at low pH or in the presence of urea. Assessment of electrophoretic homogeneity of the intact complex is, therefore, necessary in an anionic system and at alkaline pHs. Complete assurance of electrophoretic homogeneity, however, cannot be obtained at only one pH level. In addition to problems inherent in the evaluation of homogeneity of oligomeric proteins, which tend to dissociate and self-associate, other problems involved with microheterogeneities of glycoproteins have been well documented (14–16).

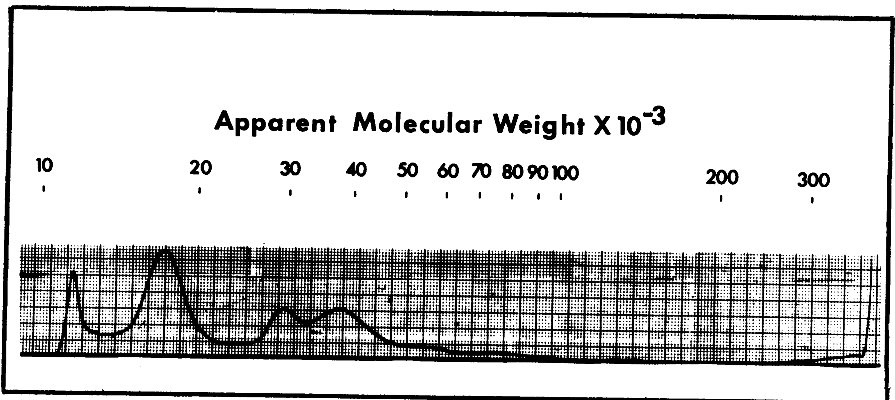

Fig. 2. Densitometric scan of SDS disc gel (12S glycoprotein). Apparent molecular weight scale was determined with standard proteins, bovine thyroglobulin, conalbumin, ovalbumin, and RNase.

Electrophoresis

Figure 1 illustrates a densitometric scan of a typical disc gel stained with Coomassie blue. At pH 9.5 (Davis system), the protein migrates only slowly and is clearly separated into a major component and a slower-moving minor component, presumably the 17S component described elsewhere (1).

Figure 2 illustrates a densitometric scan of an SDS gel in which the reduced, dissociated 12S protein was electrophoresed. Apparent molecular weights of the separable fragments were 37,300 ± 400, 30,300 ± 1,100, 17,400 ± 400, and 12,200 ± 1,000, with the limits representing standard errors of the mean of four determinations. Since SDS does not bind well to glycoproteins, however, anomalous behavior on SDS gels may sometimes occur for glycopeptide subunits (17–19).

On this basis, the stoichiometric relationship between the subunits separable by SDS gel electrophoresis is difficult to determine. The smallest subunit traveled with the bromphenol blue tracking dye, although its presence was confirmed from experiments in which no tracking dye was used. In one of the runs, the component with the highest molecular weight was clearly separated into two components with apparent molecular weights of 42,000 and 37,600 daltons.

Figure 3 (upper) illustrates a densitometric scan of the dissociated 12S

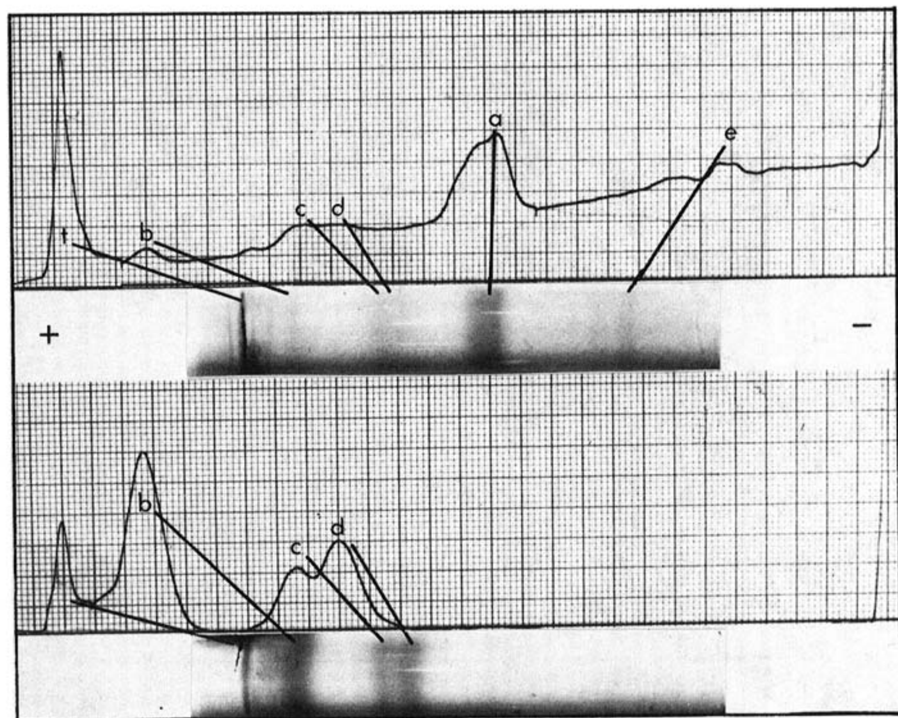

Fig. 3. Densitometric scans of SDS gels run with unreduced dissociated 12S glycoprotein (upper) and 12S protein reduced with 0.15*M* dithiothreitol (lower).

complex that was not reduced with dithiothreitol. The major component (a) of the unreduced protein had an apparent molecular weight of 60,000–70,000 daltons, although minor peaks that corresponded to the reduced fragments (b, c, and d) were observed. A high molecular weight component (e) was observed and may correspond to a small amount of unfragmented 12S protein. The lower portion of Fig. 3 demonstrates the effect of reducing agent on the fragmentation of the 12S aggregate. Although only trace amounts of 1/2 cystine were recovered in the amino acid analyses (3), intermolecular disulfide bonds are present. Similar results were observed with both dithiothreitol and 0.15*M* 2-mercaptoethanol. Goding et al (4) reported that 0.02*M* 2-mercaptoethanol had no effect on extraction, chromatography, or electrophoresis of the 12S globulin prepared from seed (*B. campestris* L. var. Echo and *B. napus* L. var. Target). MacKenzie (5), however, reported that 0.1*M* mercaptoethanol did contribute to the dissociation of the *B. juncea* 12S aggregate, although 0.05*M* mercaptoethanol had no effect. Thus, the effect that Goding et al (4) reported may have resulted from an insufficient level of disulfide-reducing reagent.

A method similar to that of Glossmann and Neville (9) was used to detect the presence of glycopeptides in SDS gels. The technique involved the removal of both bound and unbound SDS, since the detergent itself will react with Schiff reagent. Figure 4 illustrates a typical densitometric scan. The only PAS-positive fragment (g) resulting from the dissociation of the 12S aggregate migrated with the tracking dye (t), which has been marked with drafting ink before periodate

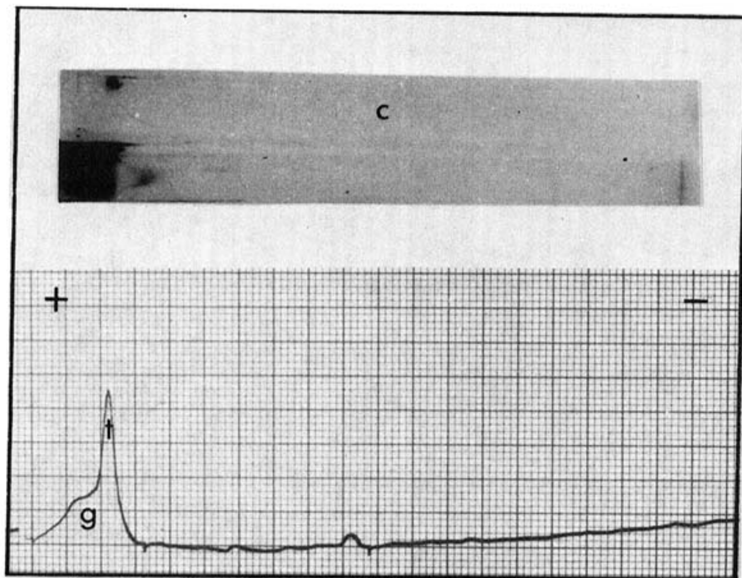

Fig. 4. Upper: Schiff-stained SDS gel showing PAS-positive fragment, which electrophoresed slightly ahead of tracking dye, compared with control gel (c), which received no periodic acid treatment. Lower: Densitometric scan of PAS-treated gel showing position of carbohydrate-containing band (g) and tracking dye (t).

oxidation and Schiff staining. A control gel that was not oxidized with periodic acid did not react. Band g was broader than the lowest molecular weight peptide (12,200 daltons) observed with Coomassie blue staining and, in some cases,

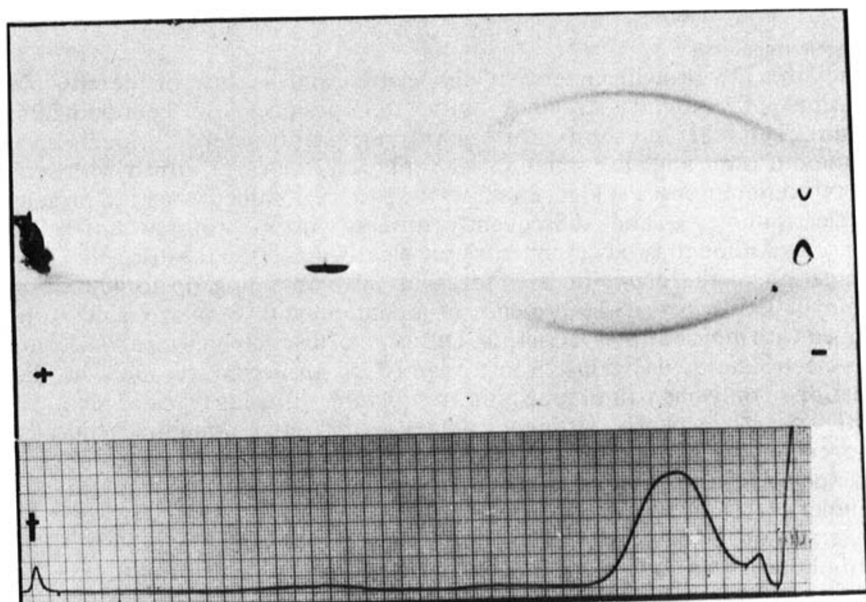

Fig. 5. Photograph of the disc immune gel pattern produced when 12S rapeseed glycoprotein was diffused against rooster anti-WRE antiserum compared with staining pattern of 12S protein run on acrylamide gel (Davis system).

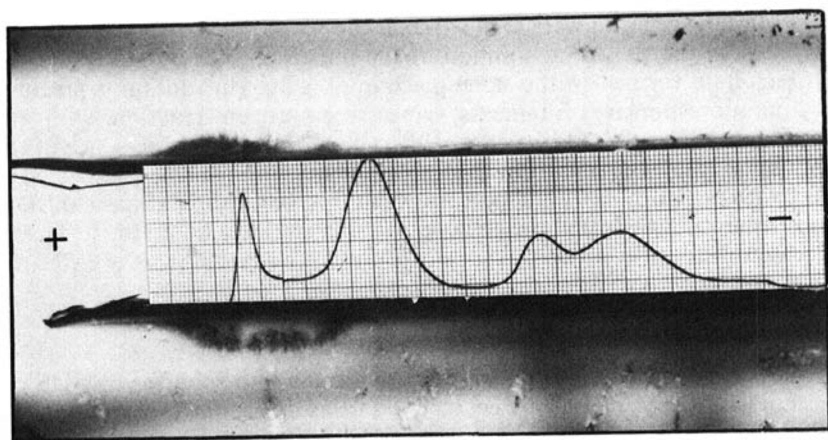

Fig. 6. Precipitin arcs formed by PAS-positive fragment of 12S protein in Ionagar No. 2 with superimposed densitometric scan of Coomassie blue-stained SDS gel to show locations of fragments. Note feathery appearance of arcs in presence of SDS.

particularly gels with large sample loads, appeared to precede the tracking dye slightly. One explanation for this observation could be that there are actually two low molecular weight peptides, one containing most of the carbohydrate and the other almost none. Since peak g contained all of the PAS-reactive material, the apparent molecular weight of 12,200 daltons may be overestimated.

Immunoelectrophoresis

Since the 12S protein underwent dissociation and because of the possible confusion of subunit structure with the presence of contaminants, immunochemical methods of characterization were undertaken. Immunodiffusion and disc immunoelectrophoresis were performed with sera from two different species. Figure 5 shows the pattern obtained when 12S protein was electrophoresed and subsequently diffused against rooster anti-WRE antiserum. Although two components were clearly present in the disc gels, close examination of the precipitin arcs (even in gels containing up to 400 μg of protein) failed to reveal the presence of a contaminant. Similar results were obtained with mouse anti-WRE serum. Diffusion of disc gels on which WRE had been electrophoresed against rooster anti-12S antiserum resulted in the formation of only one pair of precipitin arcs. Double diffusion revealed identical results. Such evidence strongly suggests that the immunochemically homogeneous preparation of the 12S glycoprotein in the present study probably self-associates to form dimers that are immunologically identical to the monomer.

Determining the degree of immune response of the peptides separable by SDS electrophoresis was interesting. Expecting the glycopeptide or glycopeptides to be more hydrophilic and therefore oriented toward the surface of the molecule in aqueous solution would seem reasonable. To test this hypothesis, SDS gels on which the dissociated 12S protein had been run were subsequently diffused against rooster anti-12S precipitating antibody. Figure 6 illustrates a plate that was stained with amido black to increase the contrast of the bands. The bands in the SDS immune gels precipitated nearer to the agar-acrylamide interface than in ordinary immune gels and were atypical since they appeared somewhat feathery, possibly due to the partial dissolution of the precipitin arcs by the SDS. The white precipitate formed in the same place in all gels. This does not preclude other immunoresponsive fragments, since the antigen reaction with the precipitating antibody would be expected to be reduced in the presence of the dissociating agent's urea and SDS. Moreover, if any fragment were expected to complex with antibody, it would be a fragment that bound a minimum of SDS. This experiment suggests that the glycopeptide may be situated on the surface of the 12S aggregate.

CONCLUSIONS

The 12S rapeseed glycoprotein has been separated into subunits by SDS gel electrophoresis. The apparent molecular weights as determined by this method were 42,000, 37,600, 30,100, 17,400, and 12,200 daltons. The 12S oligomer contained intermolecular disulfide bonds, although the cystine content was low. The 12,200-dalton subunit contained most of the PAS-reactive carbohydrate and was oriented toward the surface of the 12S complex.

Acknowledgment

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