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ISOLATION AND COMPARISON OF GLYCOPROTEIN FRACTIONS FROM BOVINE LACTEAL PROTEOSE-PEPTONE AND FAT GLOBULE MEMBRANE

By

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ABSTRACT

ISOLATION AND COMPARISON OF
GLYCOPROTEIN FRACTIONS FROM BOVINE LACTEAL
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Affinity chromatography with a Concanavalin A-Sepharose support was used to isolate glycoprotein fractions from proteose-peptone (P-P) and the soluble protein of the milk fat globule membrane (s-MFGM). The glycoprotein fractions were characterized by immunological, chemical, and electrophoretic techniques. The P-P glycoprotein fraction was shown by chemical and electrophoretic analyses to consist principally of component 3. Immuno-double diffusion experiments indicated the presence of at least one antigenically similar component in the P-P glycoprotein fraction and the s-MFGM glycoprotein fraction 1. Electrophoretic comparison (SDS-PAGE) of these two glycoprotein fractions revealed four protein zones common to both fractions, with apparent molecular weights of 18-21,000, 24,800, 28,200, and 32,200. Immunological techniques identified the 18-21,000 molecular weight glycoprotein as the antigenically common species in P-P and s-MFGM. These results suggest that the fat globule membrane is the origin for this glycoprotein in the P-P fraction.

To my mother and father for

their encouragement

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INTRODUCTION

Proteose-peptone is a minor protein fraction found in bovine milk which accounts for approximately 4% of the total skim milk protein. It is a heterogeneous group of proteins and peptides which are acid-soluble (pH 4.6) and heat-stable (95°C for 30-40 minutes). Therefore, proteose-peptone can be recovered from skim milk after removing the principal proteins, i.e. caseins and heat-denaturable whey proteins, by heating and acidification. Electrophoretic characterization has revealed four main components in proteose-peptone, which are termed components 3, 5, 8-slow, and 8-fast. Each of these principal components has been shown to be heterogeneous. Component 3 is a glycoprotein fraction which has been reported to contain 17.2% carbohydrate (Ng, Brunner, and Rhee, 1970). It is found solely in the whey protein fraction of skim milk. Components 5, 8-slow, and 8-fast, however, have been isolated from both the serum and micellar casein phases and are characterized by relatively high contents of phosphorus, thus, for a number of years it was thought that they were minor caseins. Recent studies have, in fact, demonstrated that components 5, 8-slow, and 8-fast are composed of proteolytic breakdown products of one of the major milk proteins, β-casein (Andrews, 1979; Eigel and Keenan, 1979; Jenness, 1979).

The origin of the component 3 glycoprotein fraction is unknown, however, there is some evidence that it may be derived

from the protein complement of the milk fat globule membrane, perhaps by a similar proteolytic mechanism (Brunner and Thompson, 1961; Kanno and Yamauchi, 1979). Brunner and Thompson (1961) were the first workers to suggest a possible relationship between proteose-peptone and the fat globule membrane when they noted that the soluble protein fraction of the milk fat globule membrane possessed heat stability and compositional characteristics quite similar to proteose-peptone. In addition, they noted a common major component in the free-boundary electrophoretic patterns of the two protein fractions.

The objective of this study was to explore the relationship between the proteose-peptone glycoproteins, component 3, and the soluble protein fraction of the milk fat globule membrane to determine whether the fat globule membrane is, indeed, the origin of component 3 glycoproteins. Thus, proteose-peptone component 3, as well as a glycoprotein fraction from the soluble protein of the fat globule membrane, were isolated by specific adsorption on a Concanavalin A-Sepharose support. The two glycoprotein fractions were compared by immunological, chemical, and electrophoretic means.

LITERATURE REVIEW

Historical

Osborne and Wakeman (1918) were the first workers to observe the existence of an acid-soluble, heat-stable protein fraction in bovine milk. They demonstrated that some protein material remained in solution after heat denaturation and precipitation of the albumins and globulins in acid whey. Osborne and Wakeman (1918) were uncertain as to whether this residual protein was native to milk or an artifact resulting from the heat treatment.

Palmer and Scott (1919) showed that α-lactalbumin and β-lactoglobulin were not the only proteins present in milk serum. They used tannic acid to precipitate protein from casein-free, heat-denatured whey. Somewhat later, Kieferle and Gloetzl (1930, 1933) precipitated the soluble proteins from heat-coagulated milk with phosphotungstic acid, these were classified as proteoses and peptones.

Jones and Little (1933) designated proteose as the protein precipitated by 10% trichloroacetic acid (TCA), but not 5% TCA. They reported a considerable quantity of this material in milk.

By heating a casein-free filtrate of milk, Moir (1931) demonstrated that approximately 70% of the soluble proteins were heat coagulated; while 30% remained in solution.

Rowland (1937a, 1937b) used various concentrations of TCA to fractionate the soluble proteins of a casein-free filtrate of milk and show that secondary proteins of a proteose-peptone nature are present. He (Rowland, 1938a) defined proteose-peptone as a protein fraction which is not precipitated at pH 4.7 after heating skim milk at 95°C for 30 minutes, but is precipitated by 12% TCA. In the same paper, Rowland reported improved methods for the precipitation and determination of all the milk proteins using acetic acid and sodium acetate to precipitate casein, magnesium sulfate to salt out the globulin fraction, and TCA to determine total protein and proteose-peptone. Using these improved procedures along with a semi-micro Kjeldahl method for nitrogen determination (Rowland, 1938b), Rowland (1938c) reported the nitrogen distribution in normal milk as follows: 78.5% casein N, 9.2% albumin N, 3.3% globulin N, 4.0% proteose-peptone N, and 5.0% non-protein N.

Harland and Ashworth (1945) studied the effect of heat treatment on whey proteins. They salted out casein from skim milk by saturation with sodium chloride. The whey proteins were then precipitated by acidifying the casein-free filtrate to pH 2.0 with hydrochloric acid. This method of protein fractionation gave higher casein nitrogen values and lower whey protein nitrogen values when compared to Rowland's (1938c) method for determining nitrogen distribution. The procedure of Harland and Ashworth resulted in 17.3% less whey protein nitrogen than that reported by Rowland (1938c). Furthermore, 95% of the whey proteins isolated by the sodium chloride-HCl method were coagulated by heating at 95°C for 10 minutes, while only 77% of the whey proteins isolated by Rowland's method were heat coagulated.

Aschaffenburg (1946) isolated a residual protein-like fraction from a casein-free, heat-denatured milk filtrate by adding ammonium sulfate to half-saturation. The resulting precipitate was quite surface active and was designated sigma proteose. The chemical composition of sigma proteose resembled that of casein except for a markedly reduced nitrogen content compared to casein. Heterogeneity of sigma proteose was established by free-boundary electrophoresis in phosphate buffer at pH 8.0. The fraction was separated into three components which in decreasing order of mobility accounted for 10.5%, 82.5%, and 7.0%, respectively of the total protein. Sigma proteose was examined in the ultracentrifuge by Ogston (1946). Two components were observed with molecular weights of 4,900 and 23,900, accounting for 49% and 11% of the total protein, respectively. The remaining 40% of the material could not be accounted for.

The first workers to study the relationship between the protein fraction of milk and solar-activated flavor in milk were Doan and Myers (1936) and Keeney (1947). They observed that the solar-activated flavor of milk originates in a serum component that remains following the removal of all the major milk proteins. Weinstein, Duncan, and Trout (1951a) isolated a "minor protein fraction" from heated, rennet whey by a modification of Aschaffenburg's method (1946) that was, after being photosensitized, capable of producing solar-activated flavor in homogenized milk. This "minor protein fraction" was shown to be different from sigma proteose and other milk protein fractions by elementary and amino acid analysis. The authors did not know whether the "minor protein

fraction" was native to milk, a result of abnormal functioning of the mammary glands, or possibly a milk protein heat-degradation product. Free-boundary electrophoresis of the "minor protein fraction" (Weinstein, Lillevik, Duncan, and Trout, 1951b) exhibited two components with isoelectric zones of pH 3.7 and 4.4.

Ashworth and Drueger (1951) compared the following three methods of determining the nitrogen distribution in milk: the official A.O.A.C. method, Rowland's method (1938a, 1938b, 1938c), and the sodium chloride-HCl precipitation method described by Harland and Ashworth (1945). They isolated the protein fraction which was salted out by saturation with sodium chloride, but not precipitated by Rowland's method. From examination of the phosphorus to nitrogen ratio and of the isoelectric pH, they concluded that this protein fraction was not proteose, but rather the 0.11% of casein which normally stays in solution at the isoelectric point.

Several workers have studied the effects of heat treatments on the proteose-peptone level in milk. Shahani and Sommer (1951) observed an increase in the proteose-peptone content when milk was pasteurized at 155°F for 30 minutes followed by homogenization. However, in contradiction to these results, Menefee, Overman, and Tracy (1941) reported that neither pasteurization of milk at 145°F for 30 minutes, or homogenization at normal or abnormal pressures produced any significant change in the nitrogen distribution in milk. Melachouris and Tuckey (1966) observed that the proteose-peptone content of milk decreased as temperature of heating increased. They reported a decrease in proteose-peptone nitrogen from 0.107 mg N/ml

milk to 0.064 mg N/ml after heating at 143.3°C for 2.08 seconds. Davis and White (1959) also reported that the proteose-peptone level of milk dropped as the temperature of heating increased. Ganguli, Joshi, and Bhalerao (1968) found that heating a model system consisting of proteose-peptone and micellar casein for 15 minutes in a boiling water bath resulted in a decrease in the proteose-peptone level. Substitution of the casein in the model system with β-lactoglobulin or α-lactalbumin produced no effect on the proteose-peptone level when heated. The authors suggested a possible heat-induced interaction between proteose-peptone and micellar casein.

Using a quantitative free-boundary electrophoretic procedure Larson and Rolleri (1955) observed eight peaks corresponding to the whey protein fraction of milk. They identified peaks 1, 2, 4, 6, and 7 as euglobulin, pseudoglobulin, α-lactalbumin, β-lactoglobulin, and bovine serum albumin, respectively. Peaks 3, 5, and 8 were attributed to the proteose-peptone fraction. They were the only peaks remaining after a heat treatment of 96°C for 30 minutes. Components 3, 5, and 8 had electrophoretic mobilities of -3.0, -4.5, and -7.9 x 10^{-5} cm^2 $volt^{-1}$ sec^{-1}, and they accounted for 4.6%, 8.6%, and 5.7% of the total whey protein, respectively. Larson and Rolleri (1955) were the first workers to show the presence of proteose-peptone components in unheated milk, thus suggesting that proteose-peptone is native to milk.

Jenness (1957) found a correlation coefficient of +0.82 between the quantity of protein which is salted out of skim milk by saturation with sodium chloride but is not precipitated by addition of acid to pH

4.6, and the proteose-peptone concentration in skim milk. He isolated and partially purified this protein fraction precipitated by salt but not by acid, and found it to be the principal constituent of the proteose-peptone fraction. Over 90% of the material in purified concentrates of this protein exhibited an electrophoretic mobility identical to that of proteose-peptone component 5 (Jenness, 1959). Jenness (1959) suggested that component 5 may be the heat-labile loaf volume depressant of raw milk. Volpe and Zabik (1975) separated a loaf volume depressant from whey and identified it as proteose-peptone component 5. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate revealed a molecular weight of 14-15,000 daltons. The component stained with fuchsin-sulfite dye in the gel patterns, indicating it is a glycoprotein.

Ashaffenburg and Drewry (1959) used paper electrophoresis to separate the whey proteins. Staining with bromophenol blue and washing with dilute acetic acid stained the proteose-peptone components yellow, while all other whey proteins stained blue. The yellow bands were indicative of carbohydrate. Acid filtrates of heat-treated skimmilk showed six yellow bands. One band corresponded to proteose-peptone component 5, while the other five bands corresponded to component 3. Component 8 was not identified. The same six bands were observed in ultrafiltrates of unheated milk, thus supporting the claim that proteose-peptone is native to milk.

Thompson and Brunner (1959) used chemical and chromatographic techniques to identify the carbohydrates found in the following proteins: Rowland's (1938b) proteose-peptone, the "minor protein

fraction" of Weinstein et al. (1951a), and the soluble protein fraction of the milk fat globule membrane (Herald and Brunner, 1957). Hexose, hexosamine, fucose, and sialic acid were identified. All three protein fractions were high in hexose and sialic acid. The hexoses and hexosamines present were identified by paper chromatography to be galactose, mannose, galactosamine, and glucosamine. The same three proteins listed above, along with Jenness' (1959) whey component 5 and Aschaffenburg's (1946) sigma proteose, were partially characterized by Thompson and Brunner (1960) and Brunner and Thompson (1961). All five protein fractions were low in nitrogen (10-14%), high in ash (3-7%) and phosphorus (0.6-1.5%), and contained carbohydrate. Free-boundary electrophoretic patterns exhibited heterogeneity in all of the proteins, but there appeared to be a major component in common. Ultracentrifugal studies produced similar sedimentation-velocity diagrams for the four proteose-peptone preparations, however the soluble protein fraction from the fat globule membrane showed greater heterogeneity, suggesting that it contained components different from those of the proteose-peptones.

Marier, Tessier, and Rose (1963) reported that proteose-peptone and κ-casein were the only milk proteins which contain significant quantities of sialic acid. They found that 17-28% more sialic acid was present in proteins precipitated with 12% TCA than was present in acid precipitated casein. The difference in sialic acid level could be fully accounted for by the 1.8% sialic acid in proteose-peptone.

Acid glycoproteins have been defined by Mehl, Golden, and Winzler (1949) as proteins containing carbohydrate which are negatively

charged at pH 4.5. They studied the acid glycoproteins of human plasma and divided them into M-1 and M-2 fractions. Weimer, Mehl, and Winzler (1950) reported that orosomucoid was the major, if not only component of the M-1 group. Bezkorovainy (1963) isolated an acid glycoprotein from bovine serum which exhibited an electrophoretic mobility identical to the M-2 glycoprotein of human plasma. He designated this acid glycoprotein as bovine M-2 glycoprotein. Later Bezkorovainy (1965) showed that bovine milk contains no M-1 glycoprotein (orosomucoid) and only trace amounts of the bovine M-2 glycoprotein. The major acid glycoprotein in milk was a phosphoglycoprotein, also found in colostrum, which exhibited chemical and physical properties quite similar to those of the major component of proteose-peptone. It was concluded that close relationships could exist between the colostrum glycoproteins and the proteose-peptone fraction of milk (Bezkorovainy, 1965).

The sialic acid and hexose contents of proteose-peptone and proteose were studied by Ganguli, Gupta, Joshi, and Bhalerao (1967). They reported that proteose had higher sialic acid and hexose levels than the corresponding proteose-peptone fraction. The sialic acid in both proteose-peptone and proteose was identified as a neuraminic acid derivative similar to that found in κ-casein. Hexose levels of 2.78% and 2.30% were reported for proteose and proteose-peptone, respectively.

Two methods for the quantitative determination of proteose-peptone in milk have been reported by Ganguli's group. The first method is a colorimetric procedure based upon the reaction of aromatic amino acids

with Folin-phenol reagent (Ganguli, Agarwala, and Bhalerao, 1965). The filtrate remaining after precipitation of the milk proteins with 10% acetic acid and 16% TCA was used to develop color with the Folin-phenol reagent. They (Ganguli et al., 1965) reported a value of 0.22% proteose-peptone in cow's milk. The second method as described by Joshi and Ganguli (1968) is a turbidimetric method for quantitation of proteose-peptone and proteose using 15% TCA and ammonium sulfate as precipitants, respectively. The level of peptone was calculated by the difference between the proteose-peptone and proteose concentrations. Ganguli, Gupta, Agarwala, and Bhalerao (1965) separated proteose-peptone into three components by paper electrophoresis at pH 6-10 and observed that colostrum contained a higher proteose-peptone content than milk.

The effects of storage, trypsin action, rennet action, and repeated boiling of milk on the proteose-peptone level has been examined by Ganguli's group (Ganguli, Gupta, and Bhalerao, 1966; Joshi, Ganguli, and Bhalerao, 1967; Ganguli, Joshi, Gupta, and Bhalerao, 1968; Joshi, Ganguli, and Bhalerao, 1971). Twenty-four hour storage of milk at temperatures of 25°C or below resulted in increases in the proteose-peptone and non-protein nitrogen contents, and a decrease in the whey protein content. Storage at higher temperatures led to a decrease in proteose-peptone level. Milk which had been treated with trypsin contained four to five times as much proteose-peptone as untreated milk. Trypsin released both proteose-peptone and non-protein nitrogen from acid and/or micellar casein. Trypsin-induced proteose-peptone contained four more

components than native proteose-peptone, as shown on paper electrophoresis. These new components had slower electrophoretic mobilities and were lower in sialic acid than native proteose-peptone. The action of rennet nearly doubled the proteose-peptone level in milk. When micellar casein was treated with rennet a proteose-peptone like material was released. This proteose-peptone like material was quite similar in electrophoretic pattern, sialic acid content, and gel filtration pattern on Sephadex G-75 to the native proteose-peptone in milk. Repeated boiling of milk resulted in an increase in the proteose-peptone content. Gel electrophoretic and gel filtration patterns of proteose-peptone from repeatedly boiled milk did not show any significant difference from the patterns of proteose-peptone from normal heated milk.

Gel filtration patterns of proteose-peptone and proteose on Sephadex G-50, G-75, and G-100 were reported by Joshi and Ganguli (1972a). Both protein fractions were resolved into two components, with the first peak containing sialic acid.

Jelen, Manning, and Coulter (1973) studied secondary protein removal from heat-acid deproteinated whey. They observed that thread-like aggregates formed during concentration of the deproteinated whey at pH 4.5 in a rotary evaporator. Analytical assessments showed that these aggregates consisted primarily of proteose-peptone component 5.

Bezkorovainy, Nichols, and Sly (1976) isolated proteose-peptone from both human and bovine milk. Bovine proteose-peptone contained an average of 11% carbohydrate. Three major components were observed by

gel electrophoresis in the presence of sodium dodecyl sulfate. They had molecular weights of 30,000, 18,000, and 12,000 daltons.

Brunner's laboratory has done the most detailed work on the characterization of the proteose-peptone fraction. Kolar and Brunner (1965) identified proteose-peptone component 8 as the leading band in the urea-starch or polyacrylamide gel electrophoretic patterns of proteose-peptone. They isolated component 8 from acid whey, isoelectric casein and micellar casein. Component 8 is a tenacious contaminant of κ-casein preparations and the authors suggested that it may be identical to λ-casein (Kolar and Brunner, 1965). Kolar and Brunner (1969) isolated all three proteose-peptone components from both heated and unheated milk, showing that they are indeed native to milk, and not a result of the heat processing. Component 3 was found only in the serum, whereas components 5 and 8 were isolated from both the serum and micellar casein, suggesting that these latter two components exist in a dynamic equilibrium between the casein and serum phases. Component 8 was resolved into two principal components, designated 8-fast and 8-slow, by polyacrylamide gel electrophoresis in a continuous buffer system and by gel filtration on Bio-Gel P-10 (Kolar, 1967; Kolar and Brunner, 1970). Ultracentrifugal studies revealed molecular weights for components 5, 8-fast, and 8-slow of 14,300, 4,100, and 9,900, respectively (Kolar and Brunner, 1970).

Proteose-peptone component 3 was also isolated and studied by Brunner's group (Ng, 1967; Ng and Brunner, 1967; Ng, Brunner, and Rhee, 1970). Component 3 moved as a single, slow-moving zone in polyacrylamide gel electrophoresis. Compositional analysis showed it

to be a phosphoglycoprotein with 17.2% carbohydrate and 0.5% phosphorus. The carbohydrate fraction included 7.2% hexose (galactose and mannose), 6.0% hexosamine (glucosamine and galactosamine), 1.0% fucose, and 3.0% sialic acid. Amino acid compositions of component 3 isolated from both heated and unheated milk were very similar, thus supporting the conclusion that component 3 is an indigenous milk protein. The molecular weight of component 3 was determined to be 40,800 by ultracentrifugal studies in veronal buffer (pH 8.6) containing 5M guanidine-HCl. A minimum molecular weight of 22,000 was calculated from the concentration of the limiting amino acid, tyrosine.

Kang (1971) resolved the proteose-peptone components by gel electrophoresis in both continuous and discontinuous buffer systems. Component 3 moved as a single zone in both systems. Component 5 moved as a single zone in a continuous system, however was resolved into five closely migrating bands in a discontinuous buffer system. Component 8 appeared as two closely migrating bands in discontinuous gel electrophoresis, and was resolved into 8-fast and 8-slow with a continuous buffer system.

Kasper (1978) performed an extensive electrophoretic study of proteose-peptone prepared from skimmilk, casein-free serum, and micellar casin. The three classical proteose-peptone components were all shown to be quite heterogeneous. As many as 62 bands could be accounted for by excising zones from 7.5% polyacrylamide gels and re-electrophoresing them in 17.5% gels.

Origin of Proteose-Peptone

Several workers over the years have demonstrated that the proteose-peptone fraction is native to milk, and not a result of the heat treatment used to isolate proteose-peptone (Aschaffenburg and Drewry, 1959; Kolar and Brunner, 1969, 1970; Larson and Rolleri, 1955; Ng et al., 1970). Until the recent work of Andrews (1978a, 1978b, 1978c, 1979), Jenness (1978, 1979), and Eigel and Keenan (1979) the specific origin of the individual proteose-peptone components had been unknown. These workers reported on the origin of components 5, 8-fast, and 8-slow.

The similarity of some of the proteose-peptone components to casein was first reported by Aschaffenburg (1946) who observed that the chemical composition of sigma proteose seemed to resemble that of casein. Ashworth and Druger (1951) found that the protein fraction precipitated by saturation with NaCl, but not by acidification to pH 4.6, contained a phosphorus to nitrogen ratio and an isoelectric pH resembling casein. This salt precipitated protein was later shown to consist of proteose-peptone component 5 (Jenness, 1959). Jenness (1959) was the first worker to show that component 5 was associated with casein, as well as in the serum phase. Kolar and Brunner (1969) demonstrated that components 5 and 8 exist in equilibria between micellar casein and serum and suggest that these components may be minor caseins. The same workers (Kolar and Brunner, 1970) noted that components 5 and 8 showed similarities in chemical composition to casein, namely their high content of phosphorus and, in component 5, an equivalent concentration of proline. They (Kolar and Brunner, 1970) also observed that components 5 and 8 are gradually released from the casein micelle by repeated

isoelectric precipitations of the casein. Since components 5 and 8 are recovered, in part, from skimmilk under the same conditions under which casein is isolated, Kolar and Brunner (1970) posed the question of whether these components should be classified as caseins. Earlier, McKenzie (1967) had condemned the use of the term proteose-peptone, and suggested that proteose-peptone should be considered as part of the casein complex. Jenness (1970) stated that components 5 and 8 are undoubtedly caseins since they are phosphoproteins present, in part, in the native casein micelle.

In addition to similarities between proteose-peptone and casein, relationships have been observed between proteose-peptone and bovine blood serum, and betweeen proteose-peptone and the fat globule membrane. Thompson and Brunner (1959) studied the carbohydrates of proteose-peptone, Weinstein's "minor protein fraction", and the soluble protein fraction from the milk fat globule membrane. These proteins were all high in hexose and sialic acid, which is a characteristic of blood proteins, suggesting that these milk glycoproteins may be derived from the blood. Kolar and Brunner (1969) suggested that proteose-peptone component 3, a whey glycoprotein not associated with micellar casein, may be of blood origin. Joshi, Ganguli, and Bhalerao (1970) concluded that colostrum proteose-peptone probably appears from the blood, whereas proteose-peptone in milk is likely to originate from mammary function. Kang (1971) conducted an immunological study of the interrelation between milk proteose-peptone and bovine blood serum, and found some common components, as well as some additional components not of serum origin,

namely component 5. She (Kang, 1971) also noted antigenic similarity between proteose-peptone and the fat globule membrane.

Brunner and Thompson (1961) were the first to suggest a possible relationship between proteose-peptone and the fat globule membrane. They observed that the soluble protein fraction of the milk fat globule membrane possessed heat stability and compositional characteristics quite similar to the classical proteose-peptone fraction. Furthermore, free-boundary electrophoretic patterns demonstrated a common major component between the two protein fractions.

Proteolytic breakdown of the major milk proteins has been considered as a possible source of proteose-peptone components for many years. Riel and Sommer (1955) observed a correlation between oxidized flavor development in milk and the proteose-peptone level, resistant milk showing higher proteose-peptone levels. When milk was treated with proteolytic enzymes the resistance to oxidation increased, suggesting that proteose-peptone components were being produced by enzymatic degradation of other milk proteins. Joshi *et al*. (1967) demonstrated that trypsin released proteose-peptone components from acid and micellar casein by proteolysis. The same group (Joshi and Ganguli, 1972b) later treated κ-casein with 2-mercaptoethanol and noted an increase in the proteose-peptone content. They suggested that proteose-peptone may arise as a result of selective cleavage of κ-casein at the disulfide bridges by indigenous reducing agents in the milk or mammary glands.

Babcock and Russell (1897) were the first workers to report the presence of a natural protease in bovine milk. Milk protease (plasmin) has since been isolated and its action on milk proteins studied by

several workers (Chen and Ledford, 1971; Eigel, 1977a, 1977b; Groves, Gordon, Kalan, and Jones, 1973; Kaminogawa, Yamauchi, and Tsugo, 1969; Kaminogawa, Sato, and Yamauchi, 1971; Kaminogawa and Yamauchi, 1972; Kaminogawa, Mizobuchi, and Yamauchi, 1972; Reimerdes and Klostermeyer, 1974; Snoeren and Van Riel, 1979; Yamauchi and Kaminogawa, 1972).

Groves and his coworkers (Groves, Gordon, Kalan, and Jones, 1972; Gordon, Groves, Greenberg, Jones, Kalan, Peterson, and Townend, 1972) have demonstrated that γ-casein is derived from β-casin by cleavage of the Lys_{28}-Lys_{29} bond. The γ-casein variants, thus, consist of residues 29-209 of the corresponding β-casein variant.

Reimerdes and Klostermeyer (1974) showed that milk protease is associated with the casein micelle. They suggested thatβ-casein was the component which acted as a substrate for milk proteinase.

Eigel (1977a) reported that β-casein A^2 can be broken down by plasmin resulting in the release of γ_1-A^2, γ_2-A^2, and γ_3-A caseins. He (Eigel, 1977b) also studied the effect of bovine plasmin on α_{S1}-B and κ-A caseins. κ-A casein was unaffected, however α_{S1}-B casein was degraded resulting in formation of degradation products with molecular weights of 20,500, 12,300, and 10,300 daltons. The susceptibility of the individual casein components towards proteolysis by milk protease has been reported by several workers to be in the order of: β->- α_{S1}- > κ-casein (Kaminogawa *et al.*, 1969; Chen and Ledford, 1971; Eigel, 1977b).

Kasper (1978) postulated that the trypsin like enzyme, milk protease (plasmin), found in milk could be a primary source of some of the components of the proteose-peptone fraction. In a comparison of the

amino acid compositions of components 5 and 8-fast with the known amino acid sequence of segments of β-casein, both Jenness (1978, 1979) and Kasper (1978) found a high correlation in amino acid residues and phosphorus content between component 5 and the 1-105 and 1-107 amino acid sequences of β-casein, and between component 8-fast and the 1-28 amino acid sequence of β-casein. The 1-28, 1-105, and 1-107 sequences are the amino terminal segments of β-casein released when milk protease degrades β-casein to form the γ_1, γ_2, and γ_3-caseins, respectively. Kasper (1978) also compared the amino acid composition of component 8-slow with the segments 29-105 and 29-107 of β-casein, but found a poor correlation and concluded that 8-slow does not represent these segments of β-casein. He does suggest, however, that component 8-slow may be derived from the hydrophilic region of α_s-casein through a similar proteolytic mechanism.

Andrews (1978a, 1978b, 1979) used gel filtration on Sephadex G-75 to isolate proteose-peptone component 5. Amino and carboxy terminal amino acid determination, phosphate content, amino acid composition, and peptide mapping provided conclusive evidence that component 5 represents the 1-105 and 1-107 amino acid segments of β-casein, which are released when β-casein is degraded to γ_2- and γ_3-caseins. He (Andrews, 1978c, 1979) also isolated component 8-fast and with similar techniques demonstrated that 8-fast is identical to the 1-28 amino acid sequence of β-casein, which is released during the formation of γ_1-casein by milk protease.

Eigel and Keenan (1979) used peptide mapping to conclude that proteose-peptone 8-slow is identical to β-casein fragments 29-105 and 29-107, which are produced during proteolytic breakdown of γ_1-casein

by plasmin to form γ_2- and γ_3-caseins, respectively.

Kanno and Yamauchi (1978a, b, 1979) used gel filtration to isolate a soluble glycoprotein (SGP) from the soluble protein fraction of the milk fat globule membrane. Using immunodiffusion and immunoelectrophoretic techniques with antiserum prepared against SGP and the same antiserum absorbed with whey proteins, they found identical antigenicity between SGP and the proteose-peptone fraction of whey. The anti-SGP reacting protein was concentrated in the component 3 fraction of proteose-peptone. Gel electrophoretic patterns, in the presence of sodium dodecyl sulfate, of component 3 and SGP were quite different, however there did appear to be a major glycopeptide in common. This glycopeptide exhibited a molecular weight of 20,000 daltons and seemed to cause the identical antigenicity of both protein fractions. The presence of an antigenically similar component in both the milk fat globule membrane protein fraction and the whey protein fraction had been shown earlier by Coulson and Jackson (1962). They isolated a mucoprotein fraction from the milk fat globule membrane and used it to prepare antibodies in rabbits. Immuno-doublediffusion experiments using whey protein as the antigen and the antiserum prepared against the membrane mucoprotein revealed a precipitin band which fused with a band from the mucoprotein antigen. Thus, there appeared to be a common component between the whey protein fraction and the mucoprotein from the fat globule membrane. Coulson and Jackson (1962) considered this common component to be a contaminant, however, Kanno and Yamauchi (1978b) suggest that this component may correspond to the anti-SGP reacting protein which they observed in proteose-peptone.

EXPERIMENTAL

Chemicals and Materials

The principal chemicals used in this study along with their sources are listed in the Appendix, Table A1. All chemicals used were reagent grade unless otherwise indicated. Distilled water was used in the preparation of all buffers and solutions.

The milk used in this study was obtained from Holstein cows of the Michigan State University dairy herd. Milk was collected immediately after milking and separated as soon as possible at 40-45^{o}C on a Westfalia separator.

The Concanavalin A-Sepharose affinity chromatography medium was obtained as a ready-swollen gel from Pharmacia Fine Chemicals. It contained approximately 10 mg of coupled Concanavalin A per ml of gel.

Rabbit anti-sera against proteose-peptone and whole milk fat globule membrane was prepared in our laboratory using New Zealand White rabbits.

Equipment

Equipment used regularly during the course of this study will be discussed here. Instrumentation specific for a certain experiment will be referred to in the appropriate section.

All pH measurements were performed with an Instrumentation Laboratory Inc. pH/mV Electrometer, model 245. Dialysis of protein solutions was carried out using Visking dialysis tubing from Union Carbide Company. A laboratory-constructed lyophilizer was used for freeze-drying protein solutions. Further drying of protein samples for chemical analysis was performed in a vacuum desiccator over P_2O_5 at room temperature. A top loading digital Sartorius type 3716 balance was used for most laboratory weighings, and a Sartorius type 2463 balance was used for analytical weighings.

Low speed centrifugation was done in an International Clinical or International Model U centrifuge. For intermediate speed centrifugation a Sorvall, type RC2-B, refrigerated centrifuge was used, with either the model GSA or model SS-34 rotor.

Affinity chromatography was performed in a Bio-Rad glass Econo-column with dimensions of 20 cm X 1.5 cm. The column eluate was monitored at 254 or 280 nm with an ISCO Ultraviolet Analyzer, model UA-2. An ISCO, model 1100, fraction collector was used when fraction collecting was desired. When eluates from two columns were monitored simultaneously with the same recorder, an ISCO model 580 channel alternator was used.

Spectrophotometric determinations in the visible range were carried out using a Bausch and Lomb Spectronic 21. In the UV range a Beckman DK-2A spectrophotometer equiped with 1 cm pathlength silica cells was employed.

Flat-bed gel electrophoresis was performed on a laboratory-constructed Plexiglass cell. Gel electrophoresis in

vertical cylinders was performed with an electrophoretic apparatus manufactured by Buchler Instruments. The power supply used was either a Bio-Rad model 400 or a MRA Corporation model M158 power supply. A Bio-Rad model 170 diffusion destainer was used to destain gels following staining.

Immuno-double diffusion experiments were performed in glass Petri-dishes. The antigen and antibody wells were made with a Feinberg agar gel cutter obtained from Consolidated Laboratories, Inc. The resulting pattern consisted of a center well with an 8 mm diameter and four outer wells, each with a diameter of 6 mm. The outer wells were located a distance of 7 mm from the center well. The precipitin lines were visually examined with a hand magnifying glass.

Amino acid analyses were conducted with a Beckman model 120C Amino Acid Analyzer. Pictures of electrophoretic gels and immuno-double diffusion patterns were taken with a Polaroid MP-3 Land Camera using Polaroid type 52 film.

Preparative Procedures

Proteose-Peptone

Figure 1 outlines the isolation of proteose-peptone from fresh skim milk. The isolation scheme is based upon the method reported by Kolar (1967). Approximately 2 liters of the skim milk were heated to 95-100°C for 30-40 min. After allowing the milk to cool to room temperature the pH was adjusted to pH 4.6 with 1 N HCl. The milk was then stored overnight at 4°C to allow complete coagulation. The precipitated proteins were separated from the serum by filtering through Whatman #1 filter paper. The filtrate was clear with a green

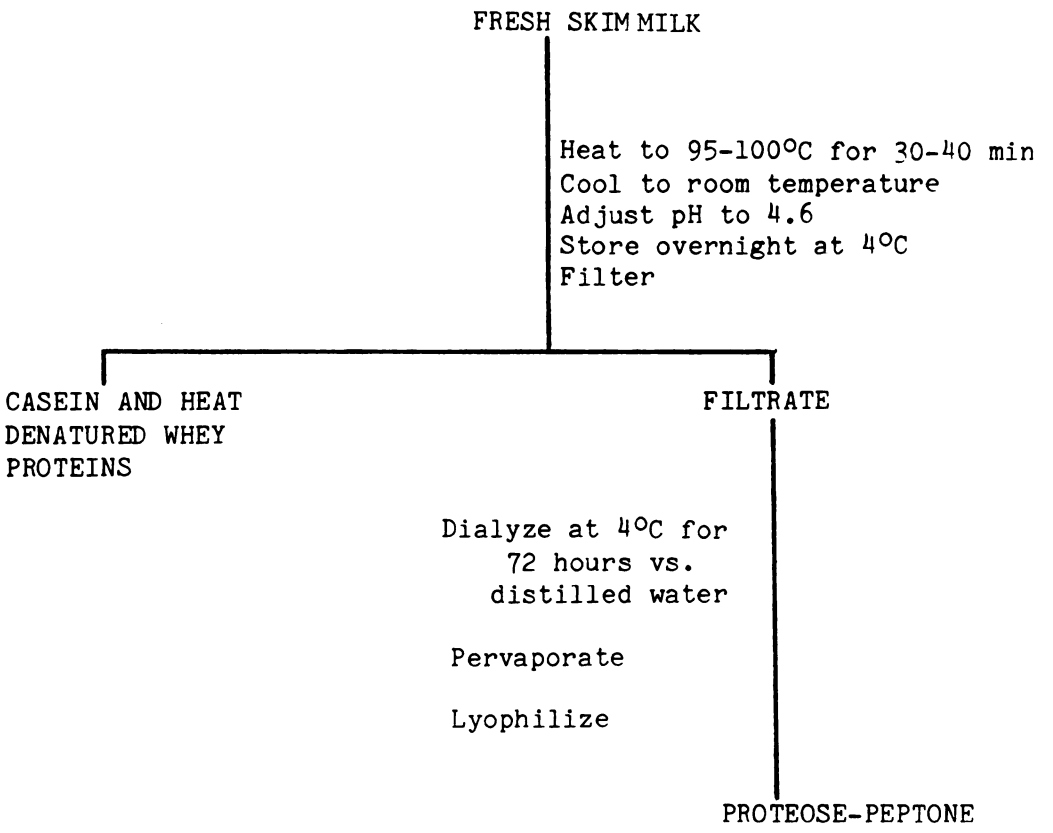


Figure 1. Procedure for the isolation of proteose-peptone from skim milk.

coloring. If necessary the filtrate was centrifuged after filtration to further clarify it. The filtrate, which contains proteose-peptone, salts, and lactose, was dialyzed against distilled, deionized water to remove salts and lactose. Dialysis was conducted at 4°C for a period of 72 h with several changes of water. The dialyzed filtrate was pervaporated and lyophilized, and stored at 0°C.

Enriched Fractions of Components 3, 5, and 8

Enriched fractions of the three proteose-peptone components were desired so that they could be used as samples in a later experiment designed to isolate the individual components by flat-bed gel electrophoresis. The enriched fractions were isolated as outlined in Figure 2 (Ng et al., 1970).

Approximately 500 mg of lyophilized proteose-peptone was dissolved in enough distilled water to give a 0.15% solution. This is the approximate concentration of proteose-peptone in skimmilk. Solid ammonium sulfate was added slowly to the proteose-peptone solution until 35% saturation was reached. This salted out an enriched component 5 fraction which was collected by centrifuging 10 min at 1,000 x g. The supernatant was brought to 55% saturation with ammonium sulfate which salted out an enriched component 3 fraction, also collected by centrifugation. The supernatant contained component 8. The enriched fractions were dispersed in distilled water and dialyzed against distilled water for 72 h, followed by pervaporation and lyophilization.

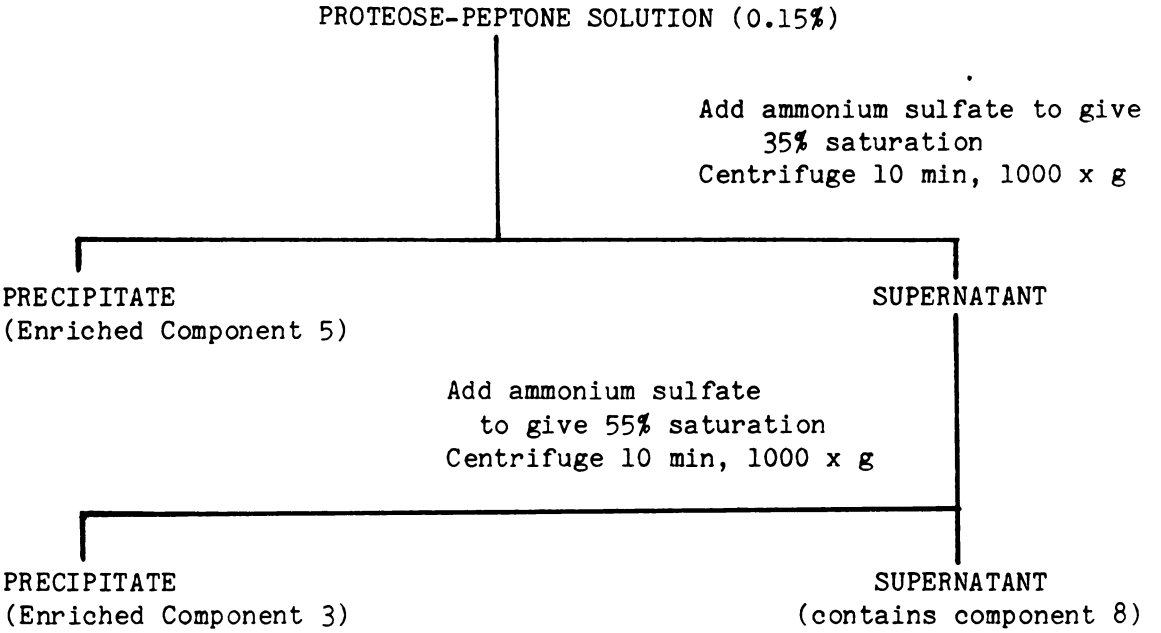


Figure 2. Isolation of enriched fractions of components 3, 5, and 8.

Soluble Protein Fraction of the Milk Fat Globule Membrane (MFGM)

The procedure described by Herald and Brunner (1957) was used to isolate a soluble protein fraction from the milk fat globule membrane (MFGM). As previously stated in the introduction, the ultimate objective of this study was a comparison of proteose-peptone with the proteins of the fat globule membrane in an effort to identify any common components. With this in mind, it was assumed that any similar components between proteose-peptone and the fat globule membrane protein would be found in the water soluble protein fraction of the fat globule membrane. Brunner and Thompson (1961) had observed an apparent common component between proteose-peptone and the soluble protein of the fat globule membrane by free-boundary electrophoresis. Thus, this was our rationale for isolating a water soluble protein fraction rather than a total MFGM protein preparation.

Figure 3 shows the isolation procedure used for preparing the soluble protein fraction of the MFGM. Approximately 10 gal of fresh whole milk was separated in a Westfalia separator at 40-45°C. The cream was collected and washed by adding three volumes of distilled water at 40-45°C followed by gentle mixing and reseparation. The cream was again collected and distilled water was added to bring the volume back to the original volume. This washing procedure was repeated three more times to ensure the complete removal of casein and whey proteins from the cream.

Following storage overnight at 4°C the washed cream was churned at room temperature. It took 40-60 min for the emulsion to break, after which churning was continued until the butter granules clumped

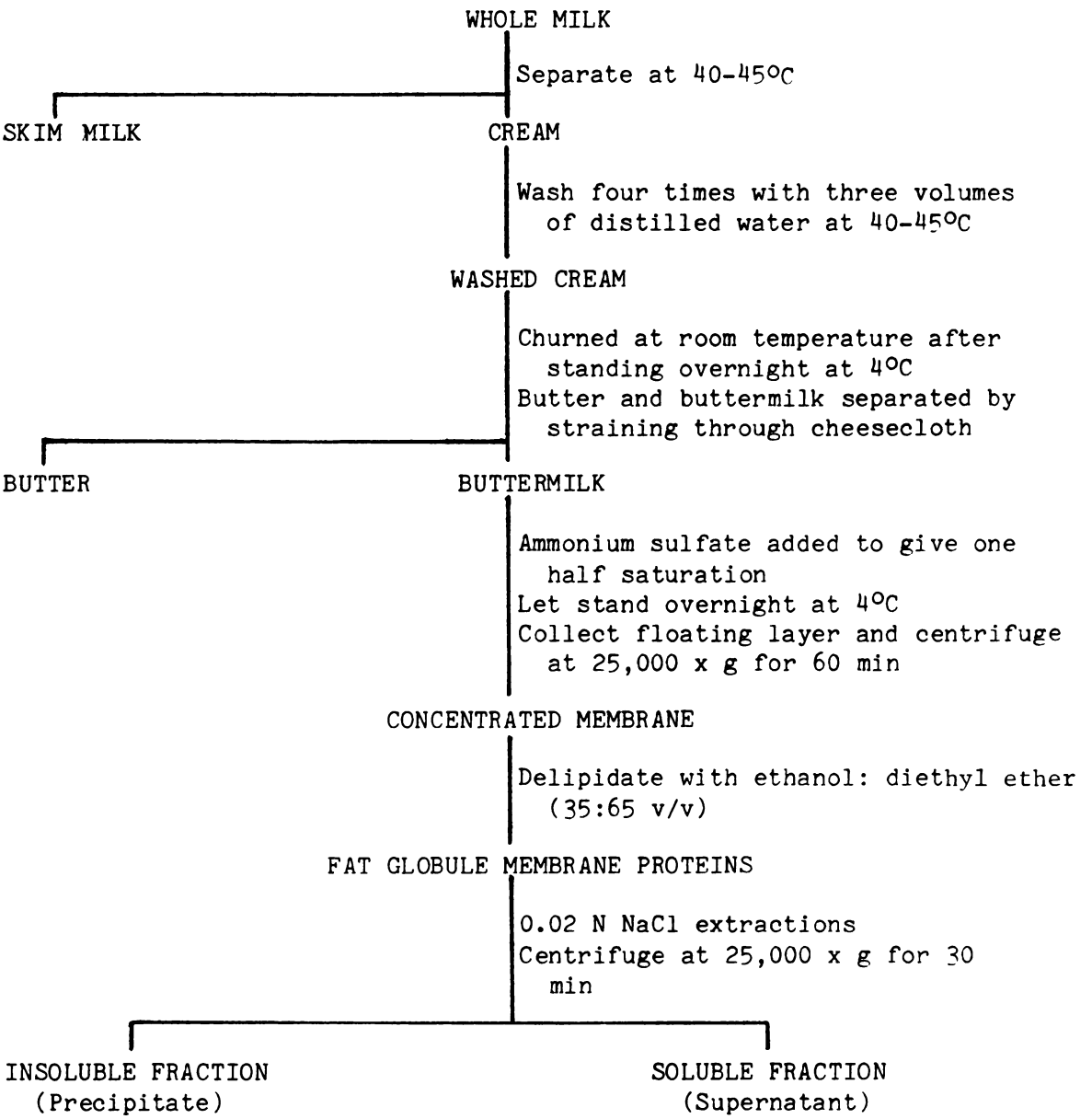


Figure 3. Procedure for the isolation of the soluble protein fraction of the MFGM.

together to form large balls. The buttermilk was separated from the butter granules by straining through cheesecloth and an equal volume of saturated ammonium sulfate solution was added to the buttermilk to give a 50%-saturated solution. The buttermilk-ammonium sulfate solution was allowed to sit overnight at 4°C in a 2 liter graduated cylinder during which the salted out membrane material formed a floating layer. The floating membrane material was collected and centrifuged at 25,000 x g for 60 minutes to concentrate the membrane material into a packed floating layer, which was reddish-brown in color. The resulting concentrated total membrane material was delipidated as follows. Five milliliters of a cold ethanol: dietyl ether mixture (35:65v/v) per gram of concentrated membrane material was added and the mixture stirred at 4°C for 60 min, followed by filtration and washing with fresh solvent. This was repeated once, after which 5 ml of cold diethyl ether was added per gram of membrane material. This mixture was stirred 1 h at 4°C and filtered. Fresh diethyl ether was added and the mixture was stirred for an additional hour at room temperature, after which it was filtered and the membrane material washed with fresh ether. After removing as much solvent as possible from the powdered total membrane protein, residual solvent was removed under vacuum. The fat globule membrane proteins were then fractionated into a soluble and an insoluble fraction by extracting with 0.02 N sodium chloride and centrifuging the mixture for 30 min at 25,000 x g. This procedure was repeated two times. The supernatants containing the soluble protein fraction were combined, dialyzed for 72 h against distilled water and lyophilized.

Affinity Chromatography

Affinity chromatography is a type of adsorption chromatography which is based on the ability of biologically active components to specifically and reversibly bind other substances, called affinity ligands. The general principle involves immobilization of an affinity ligand on an insoluble matrix and the packing of the matrix with ligand attached into a chromatographic column. A protein or enzyme sample solution can then be passed through the column and the components with affinity for the immobilized ligand will be retained while the other material will pass through unretarded. After the non-adsorbed material is washed away, desorption of the bound substance can be carried out by changing the experimental conditions to favor dissociation of the ligand-protein complex.

The affinity chromatography employed in this study involved concanavalin A covalently linked to Sepharose 4B by the cyanogen bromide method. The cyanogen bromide procedure for immobilizing proteins to insoluble supports is described by Axen, Porath, and Ernback (1967). In this study, however, the affinity matrix was purchased with concanavalin A (Con A) already immobilized. Con A-Sepharose is a group specific adsorbent for polysaccharides and glycoproteins and was used to isolate glycoprotein fractions from both proteose-peptone and the soluble protein fraction of MFGM. Con A is a lectin which shows specific binding affinity for glucose and mannose residues. For a brief discussion concerning the properties of Con A, as well as the mechanism of its interaction with glucose and mannose, see Appendix A.

Binding Study

Prior to the operation of the Con A-Sepharose affinity column a study was conducted to investigate the binding of proteose-peptone and the soluble protein fraction of MFGM to Con A. This binding study was carried out in order to determine what percentage of proteose-peptone or soluble MFGM protein was specifically adsorbed to Con A and what conditions were necessary to elute the adsorbed glycoprotein fractions.

Before performing the binding study, the Con A-Sepharose gel was washed on a coarse sintered glass filter several times with 100 ml of a 0.020 M sodium phosphate buffer (pH 7.0) containing 0.15 M sodium chloride and 0.02% sodium azide. The gel was then washed twice with 100 ml of a 5% solution of ∝-methyl-D-mannoside in the above phosphate buffer, followed by a final equilibration with the phosphate buffer. The Con A-Sepharose gel was then filtered on the sintered glass filter to remove as much buffer as possible. The semi-dry, caked gel was divided into one gram sublots and each sublot placed in a 10.0 ml test tube. Two ml of phosphate buffer was added to each test tube to resuspend the Con A-Sepharose. These one gram sublots of gel were used to perform the binding and elution studies with proteose-peptone and soluble MFGM protein.

The binding studies were performed in triplicate in the following manner. The tubes containing the gel were centrifuged at 1,000 x *g* for 10 min to sediment the gel, after which the supernatant phosphate buffer was carefully removed with a syringe. Three milliliters of the desired protein solution, with a known protein concentration and a known absorbance at 280 nm, were added to the Con A-Sepharose gel and

the mixture incubated for 30 min at 4°C with periodic gentle swirling to keep the gel suspended. The absorbance of the protein solution added to each gel sublot can be compared with the absorbances of the non-adsorbed protein eluate and the adsorbed protein eluate to determine the percentage of total protein which binds to the Con A-Sepharose matrix. Following incubation the test tubes were centrifuged at 1,000 x g for 10 min to sediment the gel. The supernatant containing the non-adsorbed protein was removed with a syringe and the absorbance read at 280 nm. To ensure complete removal of the non-adsorbed protein from the Con A-Sepharose matrix, the gel was washed with 3.0 ml aliquots of phosphate buffer until the absorbance at 280 nm was zero (2-3 washes). The total absorbance of the non-adsorbed protein fraction was calculated by adding the absorbances of the individual phosphate buffer washes. The percentage of the original total protein which did not bind to Con A was computed by dividing the total absorbance of the non-adsorbed protein by the absorbance of the original protein solution and multiplying by one hundred.

Elution of the adsorbed protein from the Con A-Sepharose matrix was carried out by washing with 3.0 ml aliquots of a 5% solution of α-methyl-D-mannopyranoside in phosphate buffer until the absorbance at 280 nm was zero. α-methyl-D-mannoside is a competitive inhibitor which binds very strongly to Con A, thus causing the release and elution of bound material. Total absorbance of the adsorbed protein is calculated by adding the absorbances of the individual mannoside washes. Percent of total protein which bound to the Con A-Sepharose matrix and was

eluted with 5%-mannoside was computed in a similar manner as for determining percent non-adsorbed protein, illustrated above. If necessary, further washes of the Con A-Sepharose matrix with higher concentrations of mannoside or other eluants was performed in an effort to release bound proteins not eluted with 5%-mannoside. When measuring the absorbances of the various washes collected in this study the reference cell of the spectrophotometer was filled with fresh eluant. This is particularly important with the mannoside washes because high concentrations of mannoside will exhibit a low amount of absorbance at 280 nm. When using the absorbances at 280 nm of the various washes to determine the percent bound and unbound protein it is important that the volume of the original protein solution as well as the individual washes are equal. In this case we used a volume of 3.0 ml.

The absorbance of proteins at 280 nm can vary depending upon the content of aromatic amino acids, therefore it is recognized that this is only a semi-quantitative method for determining protein concentration (Chung and Pomeranz, 1979). However, this procedure should give a good indication of the relative protein concentrations in the adsorbed and non-adsorbed fractions eluted from Con A-Sepharose.

Column Operation

There are many references in the literature on the use of immobilized Con A for the isolation of glycoprotein fractions, viz., Gurd and Mahler, 1974; Hunt, Bullis, and Brown, 1975; Neurath, Prince, and Lippin, 1973; Page, 1973; Schmidt-Ullrich, Wallach, and Hendricks, 1975; Susz, Hof, and Brunngraber, 1973; Westenbrink and Koornstra, 1979. We employed a Con A-Sepharose affinity column with a bed volume

of 25.0 ml. The column buffer was a 0.02 M sodium phosphate buffer (pH 7.0) containing 0.15 M sodium chloride and 0.02% sodium azide. The column was operated in the cold room at 4°C with a flow rate of 10-12 ml/hour. Protein sample which was to be applied to the Con A-Sepharose column was dissolved in the phosphate column buffer and passed through a millipore filter to remove any insoluble material. Protein loads applied to the column varied from 100-200 mg of protein dissolved in 10 ml of phosphate buffer. Following application of the sample a one to two hour incubation period was allowed before elution of non-adsorbed protein with phosphate buffer. Once the non-adsorbed protein was completely washed off the column, elution with 5%-mannoside in phosphate buffer was started in order to specifically elute the bound glycoprotein fraction. Three milliliter fractions were collected and the absorbances read at 280 nm. The fractions containing non-adsorbed protein were combined, as were the fractions containing adsorbed-5% mannoside eluted protein. The combined fractions were dialyzed against distilled water at 4°C for 72 h, pervaporated, and lyophilized.

Immunological Analysis

Preparation of Rabbit Anti-sera against Proteose-Peptone

Immune serum against proteose-peptone was obtained by immunizing New Zealand White rabbits. A desired amount of antigen was dissolved in 1.0 ml of 0.9% sodium chloride and combined with 1.0 ml of either Freund's complete adjuvant or Freund's incomplete adjuvant. A stable emulsion was produced by homogenizing the solution by repeatedly

drawing it into a syringe and forcing the solution back out through a 20 gauge needle. Injections of the emulsion were made intramuscularly into the upper thigh when complete adjuvant was used and subcutaneously on the upper back when incomplete adjuvant was used.

The rabbits were bled and the serum collected according to Cooper (1977). The ear of the rabbit was cleaned, shaved, and swabbed with toluene to dilate the peripheral vein. A single-edged razor blade was used to make a cut in the vein and approximately 25 ml of blood was collected. The blood was centrifuged for 15 min at full speed in a clinical centrifuge to sediment the red blood cells. The clear serum was removed, microbiologically stabilized with 0.02% NaN_3 and stored frozen until needed. Table 1 gives a schedule of the injections and bleedings along with the amount of antigen injected and the adjuvant used.

Antiserum against whole milk fat globule membrane, which was also used in this study, had been prepared previously in this laboratory by a similar procedure.

Immuno-Double Diffusion

A procedure adapted from Ouchterlony (1949) was used for all immuno-double diffusion experiments. Glass or plastic petri dishes were thoroughly cleaned, dried and coated with a thin layer of silicone vacuum grease. Ten to twelve ml of a 1.5% purified agar prepared in veronal buffer (pH 8.6, ionic strength=0.05) were pipetted into each dish and allowed to solidify. The antigen and antibody wells were punched in the agar with a Feinberg agar gel cutter. The agar plugs were removed with a Pasteur pipet connected to a water aspirator.

Table 1. Timetable for immunization and bleeding procedure.

Injection No.	Interval	Amount of Antigen	Route of Injection	Adjuvant	Blood Collected (ml)
1	1st day	5 mg	Intramuscular	Complete	---
2	12th day	5 mg	Intramuscular	Complete	---
3	24th day	10 mg	Subcutaneous	Incomplete	---
4	30th day	7 mg	Subcutaneous	Incomplete	---
--	34th day	---	---	---	25
---	38th day	---	---	---	25
---	42nd day	---	---	---	25
---	46th day	---	---	---	25
---	50th day	---	---	---	25
---	54th day	---	---	---	25
---	58th day	---	---	---	25
---	62nd day	---	---	---	25

Proteins which were to be tested for antigenicity were prepared in phosphate buffered saline (pH 7.0) at a concentration of 0.1%, 0.5%, or 1%. Fractions collected from the Con A-Sepharose column, which were tested for antigenicity prior to lyophilization, usually contained a protein concentration of about 1 mg/ml. After placing the antigen and antisera in the wells, the plates were incubated in a moistened chamber for 3-5 days. The precipitin bands were usually visible after two days. The plates which were to be stained and photographed were soaked in phosphate buffered saline (pH 7.0) for five days with periodic changing of the saline solution. Staining of the plates was carried out for 30-40 min with an amido black staining solution (1 g of amido black 2B, 450 ml of 1 M acetic acid, 450 ml of 1 M sodium acetate and 100 ml of glycerol). Destaining was accomplished by soaking the plates for 3-5 days in 2% acetic acid with frequent changes.

Chemical Methods

Nitrogen

Nitrogen determinations were performed in triplicate using a semi-micro Kjeldahl procedure. Samples of approximately 10 mg of dried protein were digested in 4.0 ml of a digestion mixture consisting of 5.0 g of $CuSO_4 \cdot 5H_2O$ and 5.0 g of SeO_2 in 500 ml of concentrated sulfuric acid. Digestion was carried out for one hour over a gas flame. After allowing the digestion mixture to cool to room temperature 1.0 ml of 30% H_2O_2 was added and digestion continued for another hour. Each flask was allowed to cool and the sides rinsed with distilled, deionized water. The Kjeldahl flask was then connected

to a distillation apparatus and the digestion mixture neutralized with 25.0 ml of 40% sodium hydroxide. The ammonia was steam distilled into 15.0 ml of 4% boric acid containing 4-5 drops of Kjeldahl indicator. The indicator consisted of 400 mg of bromocresol green and 40 mg of methyl red in 100 ml of 95% ethanol. Distillation was continued until 75 ml of distillate had been collected. Titration of the ammonia-borate complex was performed with 0.0206 N HCl. Standardization of the HCl was performed using Tris as a primary standard. A reagent blank and a tryptophan standard were analyzed to determine the average percent recovery of nitrogen. Average recovery ranged from 95-99%. Percent nitrogen was calculated from the following equation:

$$\%N = \frac{(V_2 - V_1)\ (\text{Normality of HCl})\ (0.01401)}{\text{grams of sample}} \times 100$$

where, V_2=ml of standard HCl used to titrate sample, and V_1=ml of standard HCl used to titrate blank.

Phosphorus

The colorimetric procedure of Sumner (1944) as modified by Swope (1968) was used to determine phosphorus content. Digestion of 10-20 mg of protein sample was carried out in 2.2 ml of 50% H_2SO_4. The samples were digested in test tubes which were heated in a sand bath to 160-170°C until the sample was completely charred. After allowing the tubes to cool to room temperature 8 drops of 30% H_2O_2 was added to each tube and heating resumed for 15 min. This was repeated until the solutions were colorless. It is extremely important to drive off all the H_2O_2 prior to continuing. The tubes were then cooled and the digestion mixture transferred to a 25.0 ml volumetric flask. Five

milliliters of a 6.6% ammonium molybdate solution was added to each tube, followed by enough distilled deionized water to give a volume of approximately 15 ml. Four milliliters of a ferrous sulfate solution was then added and the volume brought up to 25.0 ml with distilled, deionized water. The ferrous sulfate solution should be prepared immediately prior to use and is made by dissolving 5.0 g of $FeSO_4$ $7H_2O$ in 50.0 ml of distilled water, followed by the addition of 1.0 ml of 7.5 N sulfuric acid. After mixing and allowing the samples to stand for 30 min for complete color development, the absorbance was read at 660 nm. Distilled, deionized water was used as a blank. All phosphorus determinations were performed in duplicate.

A standard curve was prepared from 0.0 to 0.280 mg phosphorus using a stock solution containing 0.031 mg P/ml. The stock solution was made by diluting 10.0 ml of a 1.3609 gram KH_2PO_4/liter solution to 100 ml with distilled, deionized water.

Hexose

The determination of hexose was carried out according to the method of Dubois, Gilles, Hamilton, Rebers, and Smith (1956). A weighed amount of protein sample was placed into a test tube and 1.0 ml of distilled, deionized water added. After mixing to dissolve the sample 1.0 ml of a 5% phenol solution was added and the contents mixed. Redistilled phenol was used to prepare the 5% solution. Five milliliters of concentrated sulfuric acid were added to each tube using a fast delivery pipette with a portion of the tip removed. The stream of acid should be directed against the liquid surface and the solution agitated (vortexed) simultaneously. It is critical to the

reproducibility of this method that each tube be mixed equally. After allowing the tubes to stand 10 min at room temperature, they were again agitated (vortexed), and placed in a 25-30°C water bath for 20 min. Absorbance was read at 490 nm. A blank was prepared by omitting protein from one tube. All samples were analyzed for hexose in triplicate.

A standard curve for hexose from 0.0 to 100 µg was prepared using a 1:1 mixture of galactose and mannose.

Hexosamine

Determination of hexosamine was performed according to the procedure outlined by Cessi and Piliego (1960). Their procedure is a modification of the method developed by Elson and Morgan (1933), in which the amino sugars are reacted with acetylacetone in a hot, mildly alkaline solution. The pyrrole compounds which are produced are then reacted with p-dimethylaminobenzaldehyde (Erlich reagent) to produce a pink color. The modification employed by Cessi and Piliego (1960) involves the steam distillation of 2-methylpyrrole into Erlich reagent. 2-Methylpyrrole is a volatile compound produced among the mixture of pyrroles.

A 0.5 N sodium carbonate-sodium bicarbonate buffer (pH 9.8) containing 0.1 M sodium chloride was prepared and used to make the acetylacetone reagent. One ml of colorless, freshly redistilled acetylacetone was added to 100 ml of the sodium carbonate-sodium bicarbonate buffer. The pH was checked and adjusted to 9.8 if

necessary. The acetylacetone reagent may be stored for one day at 4°C. Erlich reagent was prepared by dissolving 80.0 mg of p-dimethylaminobenzaldehyde in 100 ml of absolute alcohol containing 3.5 ml of concentrated HCl. Erlich reagent can be stored several days at 4°C.

The protein samples were hydrolyzed to release the amino sugars as follows. Five to ten mg of sample were weighed into a 10 ml ampoule and 5.0 ml of 4N HCl added. Dissolved gases were released from the mixture by freezing in a dry ice-ethanol bath followed by slow melting under vacuum. The sample was refrozen and the ampoule sealed with a propane torch. The ampoules were placed in an oil bath in a 100°C oven for 6 h. Following hydrolysis, the ampoules were opened and the hydrolysate transferred to a 10.0 ml volumetric flask. Each ampoule was rinsed with several 1.0 ml portions of 4N NaOH which were added to the 10 ml volumetric flask. The volume was then made to 10.0 ml with 4N NaOH.

Acetylacetone was reacted with the released amino sugars by adding 5.5 ml of acetylacetone reagent to a 2.0 ml aliquot of sample hydrolysate in a small distillation flask. The mixture was heated for 20 min in a boiling water bath after which the flasks were cooled in tap water and then connected to a distillation apparatus. By heating the flasks with a bunsen burner 2 ml portions were distilled into 10 ml volumetric flasks containing 8.0 ml of Erlich reagent. The distillate-Erlich reagent mixture was allowed to stand for 30 min and then the absorbance read at 545 nm. A blank was prepared using a 2.0 ml aliquot of distilled water in place of the sample hydrolysate. All hexosamine determinations were performed in duplicate.

A standard curve from 0.0 to 62.5 μg of hexosamine was prepared with a 1:1 mixture of glucosamine-HCl and galactosamine-HCl. When weighing out the standards in order to prepare the standard curve the HCl content was taken into account so that the results can be reported as % hexosamine.

Sialic Acid

Sialic acid, after first being released from its bound form by a mild acid hydrolysis, was determined by the thiobarbituric acid method described by Warren (1959). The thiobarbituric acid method is sensitive and reproducible. It is based upon the periodate oxidation of sialic acid to form cleavage products which react with thiobarbituric acid to give a compound which exhibits maximum absorbance at 549 nm.

Ten mg of sample was accurately weighed into a test tube and 10.0 ml of 0.1 N H_2SO_4 was added. The tubes were incubated at 80°C for 30 min in a water bath. An aliquot of 0.4 ml was taken from the sample hydrolysate and pipetted into another test tube. To this 0.4 ml aliquot was added 0.1 ml of 0.2 M sodium meta-periodate in 9 M phosphoric acid. The mixture was shaken and allowed to stand at room temperature for 20 min, after which 1.0 ml of sodium arsenite solution was added. The tubes were then shaken until the yellow-brown color disappeared. The sodium arsenite solution consisted of 10% sodium arsenite in a solution of 0.5 M sodium sulfate-0.1 N H_2SO_4. The next step was the addition of 3.0 ml of 0.6% thiobarbituric acid in 0.5 M sodium sulfate. The tubes were vortexed, capped with marbles, and heated in a vigorously boiling water bath for 15 min, after which

they were removed and placed in cold water for 5 min. A 4.3 ml volume of cyclohexanone was then added to each tube, the tubes vortexed, and the contents transferred to a conical centrifuge tube. The tubes were centrifuged for 5 min in the clinical centrifuge. The upper, clear cyclohexanone layer contains the chromophore and is reddish in color. The absorbance of this phase was read at 549 nm. A blank was prepared by using 0.4 ml of distilled water as the original sample aliquot. All sialic acid assays were performed in triplicate.

The standard curve for sialic acid was prepared from 0.0 to 18.0 μg using a 45 μg/ml solution of N-acetylneuraminic acid.

Fucose

For the determination of fucose a colorimetric method was used in which the sample was heated with sulfuric acid, followed by the addition of cysteine hydrochloride to produce a green-yellow color (Dische and Shettles, 1948). The absorbance was read at two wavelengths, 396 and 430 nm, and the difference between these absorbances compared to that given by a standard fucose solution. The standard fucose solution contained 20 μg per ml of 0.1 M NaCl.

Five to ten mg of sample was dissolved in 5.0 ml of 0.1 M NaCl. Duplicate 1.0 ml aliquots of the sample solutions were pipetted into test tubes. A blank was prepared by using 1.0 ml of 0.1 M NaCl and a standard was run with 1.0 ml of the fucose standard solution. The samples, the blank, and the standard were all placed in an ice water bath for several minutes, followed by the addition of 4.5 ml of ice cold H_2SO_4-H_2O solution to each tube. The contents of the tubes were mixed while in the ice water bath. The H_2SO_4-H_2O solution

was prepared by diluting six volumes of concentrated sulfuric acid with one volume of distilled water and storing the solution in an ice water bath. After complete mixing of the samples, the tubes were transferred to a room temperature water bath for several minutes. Then the tubes were placed in a vigorously boiling water bath for exactly 3.0 min. The water should not stop boiling when the tubes are added. After heating, the tubes were transferred to the room temperature water bath for 10 min. A volume of 0.1 ml of a 3% cysteine-HCl solution was added to one of the duplicate samples and to the blank and the fucose standard, followed by mixing. Cysteine-HCl was not added to the other duplicate sample to correct for non-specific color development. All the tubes were allowed to stand at room temperature for 2 h, and then the absorbances were read at 396 and 430 nm. The absorbances of the samples to which cysteine-HCl was not added were subtracted from the absorbances of the corresponding duplicate samples to which cysteine-HCl was added. The fucose content of the samples was determined by the following equation.

$$\frac{\mu g \text{ Fucose}}{ml} = \frac{(A_{396}-A_{430})_{sample} - (A_{396}-A_{430})_{blk}}{(A_{396}-A_{430})_{std.}} \times 20.0 \frac{\mu g \text{ fucose}}{ml}$$

During measurement of the absorbances of the blank, standard, and duplicate samples distilled water was used to zero the spectrophotometer. Fucose determinations were performed in duplicate.

Amino Acids

Amino acid analyses were performed on 24 and 72 h HCl hydrolysates of protein using a Beckman Model 120C Amino Acid Analyzer (Moore, Spackman, and Stein, 1958).

Four to five mg of protein sample was accurately weighed into 10 ml hydrolysis tubes equipped with screw caps. Five ml of 6 N HCl were added to the hydrolysis tubes and then nitrogen gas was bubbled through the solution to remove gases. Hydrolysis of the samples was performed in a heating block regulated at 110°C for 24 or 72 h.

Following hydrolysis the tubes were allowed to cool and 2.5 μmoles of norleucine and 2.5 μmoles of S-B-(4-Pyridylethyl)-DL-Penicillamine were added to each hydrolysate as internal standards. The hydrolysates were then quantitatively transferred to 25.0 ml pear-shaped flasks, and evaporated to dryness on a rotary evaporator. The sample residue was redissolved in a small quantity of distilled water and again taken to dryness. This was repeated until all the HCl was removed. The dried hydrolysate was then dissolved and diluted to 5.0 ml with 0.067 M citrate-HCl buffer (pH 2.2). This solution was filtered through a 0.2 micron Millipore filter and 0.2 ml aliquots were applied to the analyzer for analysis.

Methionine and half-cystine undergo a variable amount of oxidation during acid hydrolysis, thus they must be quantitated by a different method. The procedure described by Lewis (1966) was used to estimate half-cystine as cysteic acid and methionine as methionine sulfone. This method involves the performic acid oxidation of half-cystine and methionine to cysteic acid and methionine sulfone, respectively. Methionine is quantitatively oxidized to methionine sulfone, however the yield of cysteic acid is only 94%. Therefore, the amount of cysteic acid was divided by 0.94 to give a corrected value for half-cystine content.

The determination of half-cystine and methionine is as follows. A 4 to 5 mg sample of protein was weighed into a 25 ml pear-shaped flask. Ten ml of performic acid was added and oxidation was carried out for 15 h at 4°C. After oxidation 1.0 ml of norleucine (2.5 mole/ml) was added and the performic acid evaporated on a rotary evaporator. The sample was then quantitatively transferred to a 10 ml hydrolysis tube with 5 ml of 6 N HCl. Nitrogen was bubbled through the solution to remove gases and hydrolysis performed at 110°C for 24 h. Following hydrolysis the sample was treated in a similar manner as for the acid hydrolysis.

The amino acid peaks on the chromatograms were integrated by hand-calculation according to the height-width method or the absorbance method. The amino acid composition was expressed as mole %, moles of residue per 100 moles of total residues.

Tryptophan

Determination of tryptophan was performed according to procedure W described by Spies (1967) in which the protein was first hydrolyzed with pronase.

Three to five mg of protein sample were accurately weighed into 2 ml vials, followed by the addition of 0.1 ml of pronase solution (10 mg pronase per ml of 0.1 M sodium phosphate buffer, pH 7.5). The vials were capped, agitated to mix the contents, and incubated at 40°C for 24 h. Following incubation the vials were placed in crushed ice and 0.9 ml of phosphate buffer added to each vial. The vials were then placed into 50 ml flasks containing 30 mg of p-dimethylaminobenzaldehyde and 9.0 ml of 21.2 N H_2SO_4. The flasks were agitated so as to

thoroughly mix the contents, covered with Parafilm, and allowed to stand in the dark at room temperature for 6 h. The final step is the addition of 0.1 ml of 0.045% sodium nitrite to each flask. After allowing the flasks to stand at room temperature for 30 min the absorbances were read at 590 nm.

A tryptophan analysis was also run on the pronase digestion solution, without added protein. The tryptophan content of the pronase solution was subtracted from the total tryptophan to give a corrected value for the protein samples. A blank was prepared which contained everything except protein and pronase. A standard curve was prepared from 0-120 μg with commercially available tryptophan. All analyses were performed in triplicate. The tryptophan content of the analyzed samples was combined with the amino acid data and recalculated into mole percent.

Biuret Determination of Proteins

For rapid determination of protein contents in the various Con A-Sepharose eluates, as well as in other situations when a quick but not highly accurate quantitation of protein was required, the Biuret method was employed (Cooper, 1977). Biuret reagent was prepared by dissolving 1.50 g of $CuSO_4 \cdot 5H_2O$ and 6.00 g of sodium potassium tartrate in 500 ml of distilled water, followed by the addition of 300 ml of 10% sodium hydroxide under vigorous stirring. The volume was made to 1.0 liter with distilled water. The reagent was stored in a plastic bottle and used until a black precipitate formed at which time it was discarded.

Assay for protein went as follows. One ml of protein solution, or a smaller volume adjusted to 1.0 ml with distilled water, was pipetted into a test tube and 4.0 ml of Biuret reagent added. After mixing the contents, the tubes were incubated in a water bath at 37°C for 20 min. Absorbance was measured at 540 nm. A standard curve was prepared using a 10 mg/ml solution of bovine serum albumin.

Physical Methods

Flat-Bed Gel Electrophoresis

Flat-bed electrophoresis was performed in order to isolate the individual proteose-peptone components as they are defined electrophoretically. Electrophoresis was carried out according to procedures which have been previously reported from this laboratory (Ng, 1967; Kolar, 1967).

The buffer system used was a discontinuous one consisting of borate buffer (pH 8.6) and Tris-citrate buffer (pH 8.6). A borate stock solution was prepared by dissolving 88.1 g boric acid and 19.0 g sodium hydroxide in enough distilled water to give 1.90 liter. Borate buffer was made by diluting two parts of the stock solution with three parts distilled water. A Tris-citrate stock solution was prepared by dissolving 92.0 g Tris and 12.05 g citric acid in distilled water and bringing the volume to 1.0 liter. Tris-citrate buffer was made with one part stock solution and nine parts distilled water.

An 8% total acrylamide gel (8% T) containing 5% crosslinker (5%C) was prepared by dissolving 11.4 g of acrylamide and 0.6 g of bisacrylamide in 148 ml of Tris-citrate buffer. While stirring the

gel, 0.1 ml of TEMED (N, N, N^1, N^1-tetramethylethylenediamine) and 2.0 ml of 10% ammonium persulfate were added. The gel was poured into the flat-bed apparatus and a continuous slot former was positioned 3 cm from the cathodic end. The gel was then allowed to polymerize for about 30 min under nitrogen, after which the slot former was carefully removed.

The sample to be subjected to electrophoresis was either proteose-peptone or one of the enriched component fractions and was prepared by dissolving 125 g of protein in 2.5 ml of borate buffer. Two to three drops of a 1% bromophenol blue solution were added as a marker dye and the sample applied to the sample slot in the gel. After filling the buffer reservoirs with borate buffer and connecting the electrodes to the power source, electrophoresis was started. Electrophoresis was conducted with water-cooling at a constant voltage of 110 volts so as to give an initial current of 10-12 mA/cm^2 of gel cross-sectional area. The flat-bed apparatus was carefully covered with saran wrap to reduce evaporation from the gel. It took approximately 5 h for the marker dye to migrate to the end of the gel, at which time the power was turned off.

The protein bands were visualized by cutting out 2 cm wide strips of gel from both sides of the apparatus and staining the strips in amido black stain. The stain was prepared as described earler in this section. Once the protein zones were stained, cross-sectional strips corresponding to the zones were cut out of the unstained gel. The protein components were extracted from the gel slices by macerating the

gel in a Waring blendor with distilled water. The resulting gel slurry was stirred at 4°C overnight and then centrifuged at 1,000 x g for 20 min to sediment the gel. The gel was extracted two additional times and the supernatants combined, adjusted to pH 6.5, dialyzed, pervaporated and lyophilized.

Discontinuous Polyacrylamide Gel Electrophoresis (Disc-PAGE)

Disc-PAGE was run according to the method of Melachouris (1969) with certain modifications. Electrophoresis was conducted in glass tubes with a length of 7.5 cm and an inner diameter of 6 mm. The tubes were cleaned prior to use by washing with soap and water, soaking in chromic acid overnight, rinsing with distilled water, and treating with Photo-Flo (1:200).

A separation gel stock solution was prepared by dissolving 19.00 g of acrylamide and 1.00 g of bisacrylamide in 75 ml of 0.380 M Tris-HCl buffer (pH 8.9) and made to 100 ml with the same buffer. This gave a stock solution with 20% total acrylamide and 5% crosslinker (bisacrylamide) from which gels of a desired concentration could be prepared by combining the appropriate volumes of stock solution and Tris-HCl (PH 8.9) buffer to give a final volume of 25.0 ml. To this gel solution was added 20 µl of TEMED and 50 µl of 10% ammonium persulfate. The gel was then transferred to the glass tubes with a syringe and needle, overlayed with water, and allowed to polymerize overnight. After polymerization the water layer was removed and a spacer gel layered on top of the separation gel. The spacer gel contained a total acrylamide concentration of 5% and a crosslinker

(bisacrylamide) concentration of 20%, and was prepared in a 0.062 M Tris-HCl buffer (pH 6.7). The spacer gel was also overlayed with water and allowed to polymerize for one hour.

After transferring the tubes to the electrophoretic apparatus the anodic and cathodic buffer reservoirs were filled with 0.046 M Tris-glycine buffer (pH 8.3). The protein samples were then carefully layered on top of the spacer gels in volumes of 20-50 µl. The samples were prepared as 1% solutions in the Tris-HCl (pH 6.7) buffer and to each sample sucrose and bromophenol blue marker dye were added. Electrophoresis was initially conducted at 1 mA/tube until the samples entered the spacer gel, after which a constant current of 3 mA/tube was maintained until the marker dye migrated to the bottom of the gels. After electrophoresis the gels were removed from the glass tubes and stained for either protein or carbohydrate according to one of the procedures discussed in Appendix B.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Polyacrylamide gel electrophoresis in the presence of the anionic detergent sodium dodecyl sulfate (SDS) was performed according to the procedure of Weber and Osborn (1969). Gels of 10.0 % total acrylamide (%T) with 2.63% bisacrylamide (%C) concentration were prepared in 0.1 M phosphate buffer (pH 7.2) containing 0.1% SDS. They were polymerized in glass tubes using ammonium persulfate and TEMED, as above. Protein samples were prepared as 1.0% solutions in 0.01 M phosphate buffer (pH 7.0) containing 3% SDS and 1% 2-mercaptoethanol (Kasper, 1978). Samples were incubated in a boiling water bath for 5-10 min, followed

by cooling to room temperature and the addition of sucrose and bromophenol blue marker dye. Electrode buffer was a 0.1 M phosphate buffer (pH 7.2) containing 0.1% SDS. Sample volumes of 10-20 µl were applied to the top of each gel.

Electrophoresis was performed at a constant current of 8 mA/tube with water cooling until the marker dye migrated to the end of the gel, approximately 4-5 h. The gels were then removed from the tubes, the marker dye migration distance recorded, and staining performed by one of the methods outlined in Appendix B. When it was desired to stain for carbohydrate, greater sample loads were applied to the gels (30-50 µl). This was because the carbohydrate stain of Zacharius, Zell, Morrison, and Woodlock (1969) appeared to be less sensitive than the protein stains used.

A standard curve of log molecular weight vs. relative mobility was constructed using a calibration kit obtained from Pharmacia. The kit contained phosphorylase b (94,000); bovine serum albumin (67,000); ovalbumin (43,000); carbonic anhydrase (30,000); soybean trypsin inhibitor (20,100); and α-lactalbumin (14,400). Relative mobilities of standard proteins and samples were calculated from the following equation.

$$\text{Relative Mobility } (R_f) = \frac{\text{distance protein migrated}}{\text{distance dye migrated}} \times \frac{\text{length of gel before staining}}{\text{length of gel after destaining}}$$

RESULTS AND DISCUSSION

For the sake of clarity, abbreviations for protein fractions will be used in the ensuing discussion. Proteose-peptone will be referred to as P-P, and the soluble protein of the milk fat globule membrane will be designated s-MFGM.

Affinity Chromatography

Proteose-peptone components 5, 8-slow, and 8-fast have recently been shown to be composed of proteolytic breakdown products of ß-casein (Andrews, 1979; Eigel and Keenan, 1979; Jenness, 1979). The origin of the P-P component 3 fraction has not been conclusively established, however, there is evidence that this glycoprotein fraction may be derived from s-MFGM (Brunner and Thompson, 1961; Kanno and Yamauchi, 1979). With this in mind, glycoprotein fractions were isolated from P-P and s-MFGM by Con A-Sepharose affinity chromatography.

Prior to the operation of the affinity column a binding study was conducted to determine the percentage of P-P and s-MFGM which specifically binds to Con A-Sepharose, as well as the conditions necessary to elute the bound glycoprotein fractions. The results obtained for both preparations are summarized in Table 2. For P-P, 66% of the applied protein did not bind to Con A-Sepharose and was eluted with phosphate buffer. This fraction was designated the P-P non-adsorbed fraction. The remainder of the applied P-P, which adsorbed to Con A-Sepharose and was eluted with a 5% mannoside eluant, was designated the P-P glycoprotein fraction.

Table 2. Con A-Sepharose binding and elution data[a] for P-P and s-MFGM.

	P-P	s-MFGM
Absorbance[b] of protein solution applied to gel:	1.84	1.33
Total absorbance[b] of phosphate buffer washes[c]:	1.21	0.28
Percent of applied protein which did NOT bind to Con A-Sepharose:	65.8%	21.1%
Total absorbance[b] of 5%-mannoside (in phosphate buffer) washes:	0.61	0.64
Percent of applied protein which bound to Con A-Sepharose and eluted with 5%-mannoside:	33.2%	48.1%
Total absorbance[b] of 20%-mannoside (in phosphate buffer) washes:	----	0.14
Percent of applied protein which bound to Con A-Sepharose and eluted with 20%-mannoside:	----	10.5%
Net absorbance of all eluants:	1.21+0.61=1.82	0.28+0.64+0.14=1.06
Percent recovery:	98.9%	79.7%

[a]Average values for triplicate determinations.

[b]Absorbance at 280 nm.

[c]0.02 M sodium phosphate (pH 7.0) containing 0.15 M NaCl, 0.02% NaN_3.

The s-MFGM was fractionated into three fractions by affinity chromatography (see Table 2); a non-adsorbed fraction (eluted with phosphate buffer) which accounted for 21% of the applied protein, a glycoprotein fraction 1 (eluted with 5% mannoside) which contained 48% of the applied protein, and a glycoprotein fraction 2 (eluted with 20% mannoside) which accounted for 10% of the applied protein. These three fractions will henceforth be referred to as the s-MFGM non-adsorbed fraction, s-MFGM glycoprotein fraction 1, and s-MFGM glycoprotein fraction 2, respectively. The net recovery of the three s-MFGM fractions was only about 80%, thus, 20% of the applied s-MFGM bound to the Con A-Sepharose and was not eluted with mannoside. In an effort to release the bound protein fraction which was not eluted with mannoside, a 0.1 M sodium borate buffer (pH 7.6) was employed as an eluant, with no additional release of bound proteins. Borate buffer has been reported to be effective in releasing strongly bound substances which are not eluted by the addition of sugars (Azuma, Kashimura, and Komano, 1976). Due to the lack of a suitable eluant, the irreversibly bound protein fraction was not collected or characterized. It is possible that this protein fraction may be binding to Con A through a non-specific hydrophobic interaction. This type of interaction has been reported to occur between Con A and human interferon (Davey, Huang, Sulkowski, and Carter, 1974; Davey, Sulkowski, and Carter, 1976). If, indeed, a hydrophobic interaction is occuring ethylene glycol may be an effective eluant.

The chromatogram presented as Figure 4 illustrates the elution profile for P-P passed over the Con A-Sepharose column. The first

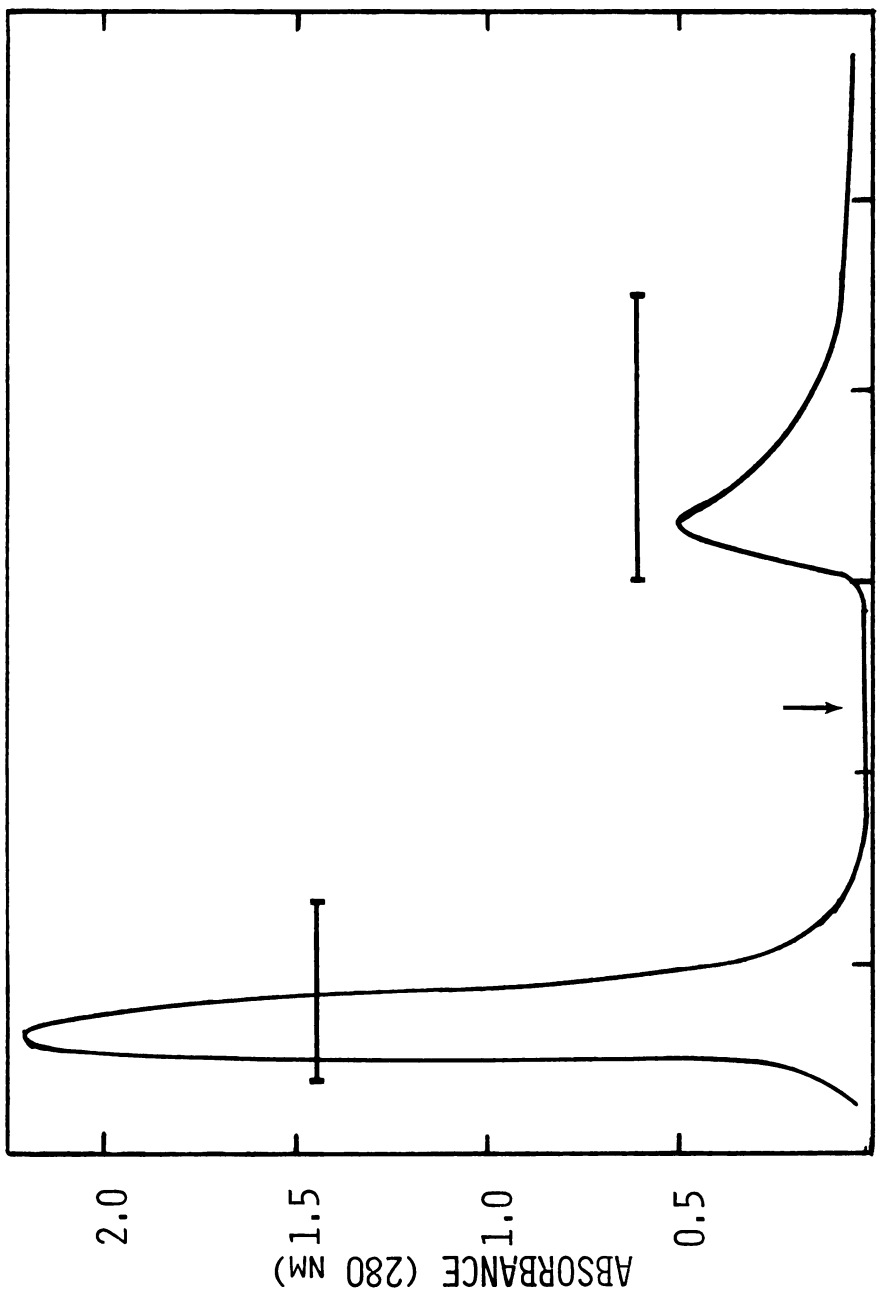


Figure 4. Elution profile for P-P on a Con A-Sepharose affinity column. The arrow signifies the point at which elution with 5% mannoside was begun. The two peaks, from left to right, are the P-P non-adsorbed and P-P glycoprotein fractions.

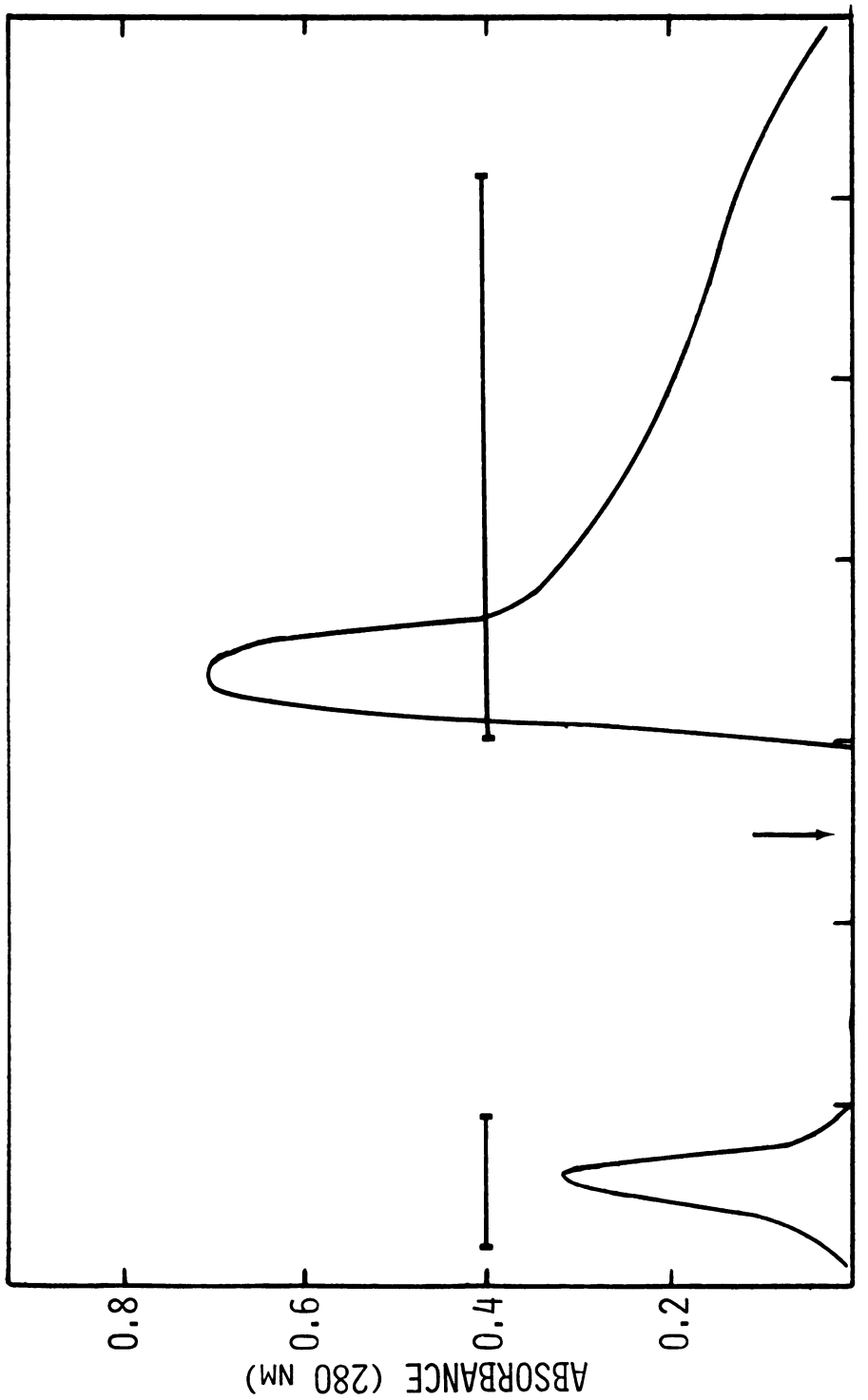


Figure 5. Elution profile for s-MFGM on a Con A-Sepharose affinity column. The arrow signifies the point at which elution with 5% mannoside was begun. The two peaks, from left to right, are the s-MFGM non-adsorbed fraction and the s-MFGM glycoprotein fraction 1.

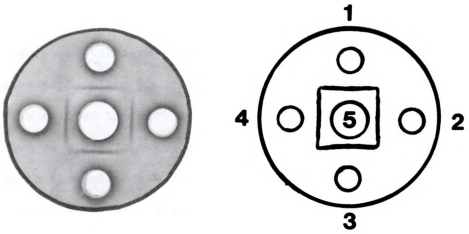
protein peak, eluted with phosphate buffer, is the P-P non-adsorbed fraction, and the second peak, eluted with 5% mannoside, is the P-P glycoprotein fraction. The elution profile for s-MFGM is represented by the chromatogram in Figure 5. The protein peak eluted with phosphate buffer is the s-MFGM non-adsorbed fraction, and the 5% mannoside eluted protein peak is the s-MFGM glycoprotein fraction 1. Though not shown in Figure 5, elution with 20% mannoside resulted in the release of the s-MFGM glycoprotein fraction 2. The non-adsorbed and glycoprotein fractions from P-P and s-MFGM were collected, lyophilized, and subsequently characterized by immunological, chemical, and electrophoretic assays.

Immunological Analysis

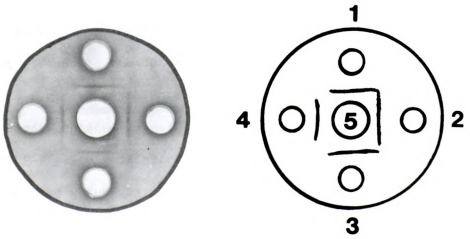
An immunological analysis of P-P, s-MFGM, and the fractions collected from the Con A-Sepharose column was conducted using the Ouchterlony double-diffusion technique. The anti-sera used were anti-proteose-peptone and anti-MFGM. Photographs and drawings of selected immuno-double diffusion patterns are presented in Figures 6, 7, 8 and 9. Certain precipitin bands which were visualized with the unaided eye are not clearly observable in the photographs. Thus, facsimiles of these bands are included in the drawings.

Kang (1971) demonstrated that there is antigenic similarity between P-P and MFGM. Immuno patterns in Figure 6 show that the antigenic similarity is in the water soluble protein fraction of the membrane, i.e., the s-MFGM. The two patterns in Figure 6 (A and B) represent separate preparations of s-MFGM reacted against anti-proteose-peptone. In both patterns strong precipitin bands developed, signifying the

Figure 6. Immuno-double diffusion patterns. Plate A: 1, 2, 3, 4 -- s-MFGM; 5 -- anti-proteose-peptone. Plate B: the same as plate A except that a different preparation of s-MFGM was used.



A



B

Figure 6

presence of at least one antigenically similar component in P-P and s-MFGM. The objective of this study, as stated previously, was to identify and characterize the components in P-P and s-MFGM which are antigenically similar.

Three immuno patterns representing various antigens reacted against anti-proteose-peptone are presented in Figure 7. When P-P (2% w/v solution) was allowed to react against its specific antiserum, the pattern shown in plate A developed. Two relatively dominant precipitin bands close to the antibody well and two faint bands close to the antigen well are apparent. Plate B shows the pattern obtained using P-P, enriched component 3, and whey as antigens. Enriched component 3 (wells 2 and 3) produced a broad precipitin band which fused with the most dominant band obtained with P-P (well 1). Thus, of the two dominant precipitin bands observed with P-P antigen (see plate A or plate B, well 1), the band closest to the antibody well is related to component 3, whereas the outer band may relate to component 5 or 8. Plate C in Figure 7 represents the pattern which developed by reacting anti-proteose-peptone against the Con A-Sepharose fractions derived from P-P. The P-P glycoprotein fraction (wells 1 and 2) produced a broad precipitin band which was identical to the dominant band attributed to P-P component 3. This was not surprising since component 3 is highly glycosylated and was expected to be found in the P-P glycoprotein fraction. The P-P non-adsorbed fraction (wells 3 and 4) produced a precipitin band identical to the other dominant band obtained with P-P antigen. This particular band could arise from either component 5 or 8, both of which would be expected to be found in the P-P non-adsorbed

Figure 7. Immuno-double diffusion patterns. Plate A: 1, 2, 3, 4 -- 2% P-P; 5 -- anti-proteose-peptone. Plate B: 1 -- 1% P-P; 2, 3, -- 1% enriched component 3; 4 -- 1% whey; 5 -- anti-proteose-peptone. Plate C: 1, 2 -- P-P glycoprotein fraction; 3, 4 -- P-P non-adsorbed fraction; 5 -- anti-proteose-peptone.

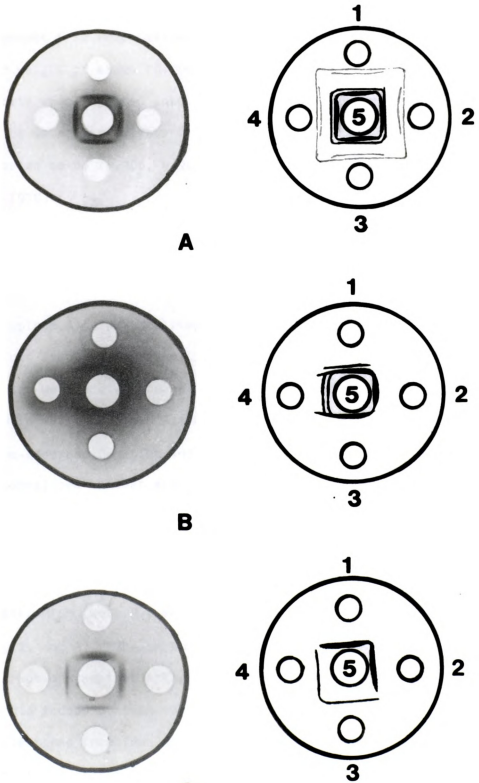
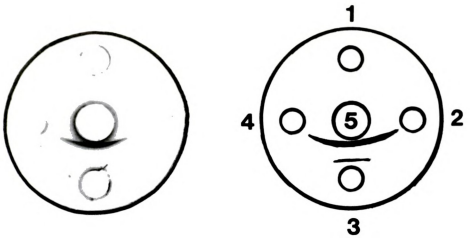


Figure 7

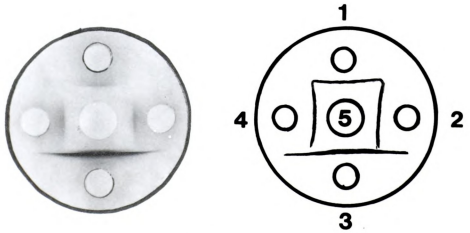
fraction on account of their low carbohydrate contents. It is likely that component 5 is responsible for this precipitin band since a minimum molecular weight of about 10,000 is required for a protein to exhibit antigenicity when injected into a rabbit (Brewer, Pesce, and Ashworth, 1974). The molecular weights for components 5, 8-slow, and 8-fast have been reported to be 14,300, 9,900, and 4,100, respectively (Kolar and Brunner, 1970).

Figure 8 represents double-diffusion patterns produced by the three Con A-Sepharose fractions derived from s-MFGM. The protein fractions were placed in the center well (well 5) as follows: s-MFGM non-adsorbed fraction (plate A), s-MFGM glycoprotein fraction 1 (plate B), and s-MFGM glycoprotein fraction 2 (Plate C). The immune sera employed in all three plates were anti-proteose-peptone in wells 1, 2, and 4, and anti-MFGM in well 3. Plate A shows a strong precipitin band between the s-MFGM non-adsorbed fraction and anti-MFGM (well 3), as was expected. No bands developed against anti-proteose-peptone. However, the s-MFGM glycoprotein fraction 1 (plate B) developed precipitin bands against both anti-MFGM (well 3) and anti-proteose-peptone (wells 1, 2, and 4). The s-MFGM glycoprotein fraction 2 (plate C) developed a precipitin band against anti-MFGM (well 3), but did not react with anti-proteose-peptone (wells 1, 2, and 4). The purpose in reacting each of the three s-MFGM protein fractions against anti-MFGM was to demonstrate that each fraction did indeed contain membrane proteins. Similarly, the purpose in reacting these fractions against anti-proteose-peptone was to ascertain which fraction contained the protein or proteins antigenically common to P-P. The double-diffusion patterns in Figure 8 clearly

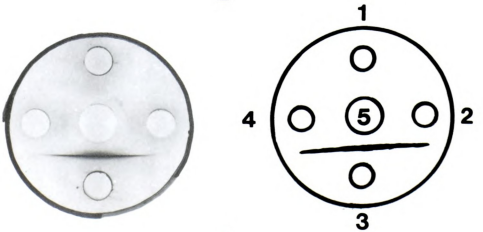
Figure 8. Immuno-double diffusion patterns. Plates A, B, and C all contain the following immune sera in the outer wells: 1, 2, 4 -- anti-proteose-peptone; 3 -- anti-MFGM. The antigens (placed in well 5) are as follows; Plate A: s-MFGM non-adsorbed fraction. Plate B: s-MFGM glycoprotein fraction 1. Plate C: s-MFGM glycoprotein fraction 2.



A



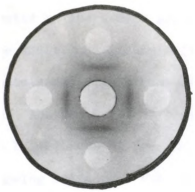
B

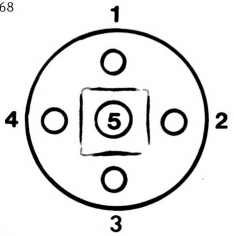


C

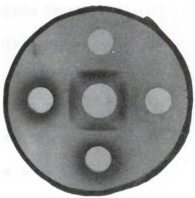
Figure 8

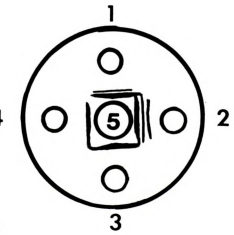
Figure 9. Immuno-double diffusion patterns. Plate A: 1, 2, 3, 4 -- s-MFGM glycoprotein fraction 1; 5 -- anti-proteose-peptone. Plate B: 1 -- 1% P-P; 2 -- 1% P-P glycoprotein fraction; 3, 4 -- 1% s-MFGM glycoprotein fraction 1; 5 -- anti-proteose-peptone. Plate C: 1 -- 1% s-MFGM; 2 -- 1% s-MFGM non-adsorbed fraction; 3 -- 1% P-P non-adsorbed fraction; 4 -- 1% P-P; 5 -- anti-proteose-peptone.



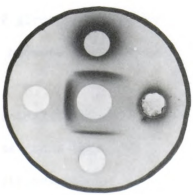


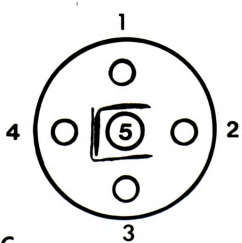
A





B





C

Figure 9

demonstrate that the species reacting against anti-proteose-peptone is concentrated in the s-MFGM glycoprotein fraction 1 (plate B).

Three additional immuno patterns are presented in Figure 9, all of which contained anti-proteose-peptone immune serum in the center well (well 5). Antigen wells in plate A contained s-MFGM glycoprotein fraction 1, which developed strong precipitin bands. Plate B contained the following antigens: P-P (well 1), P-P glycoprotein fraction (well 2), and s-MFGM glycoprotein fraction 1 (wells 3 and 4). Note the arc of identity between the P-P glycoprotein fraction (well 2) and the s-MFGM glycoprotein fraction 1 (well 3). These two glycoprotein fractions apparently contain at least one common component. Plate C in Figure 9 contains s-MFGM, P-P, and the non-adsorbed fractions from both preparations as antigens. The s-MFGM reacted strongly with anti-proteose-peptone (well 1).

Three important deductions obtained from these immunological assays are as follows: 1) there is an antigenically common component in s-MFGM and P-P, 2) the antigenically common component binds to Con A-Sepharose and is selectively eluted with 5% mannoside (i.e., the component is in the P-P glycoprotein fraction and the s-MFGM glycoprotein fraction 1), and 3) because this component binds to Con A, it is a glycoprotein.

Chemical Composition

Chemical characteristics of P-P, s-MFGM, and the fractions collected from the Con A-Sepharose column are summarized in Table 3.

Proteose-peptone contained 12.43% nitrogen, 10.04% carbohydrate, and 0.94% phosphorus. Brunner and Thompson (1961) reported a higher nitrogen level for P-P (13.70-13.90%), however, the phosphorus and

Table 3. Chemical composition of P-P, s-MFGM, and the Con A-Sepharose fractions from both preparations.

Protein Fraction	Chemical composition (%) Nitrogen	Hexose	Hexosamine	Sialic acid	Fucose	Carbohydrate (total)	Phosphorus
P-P	12.43	3.59	3.37	2.66	0.42	10.04	0.94
P-P glycoprotein fraction	10.08	6.79	7.12	3.97	0.83	18.71	0.59
P-P non-adsorbed fraction	13.45	2.93	0.91	1.60	0.18	5.62	1.07
s-MFGM	11.99	3.03	3.59	2.19	0.59	9.40	0.17
s-MFGM glycoprotein fraction 1	10.00	6.22	4.92	2.69	0.90	14.73	1.02
s-MFGM glycoprotein fraction 2	---	4.33	2.07	1.72	0.64	8.76	---
s-MFGM non-adsorbed fraction	13.05	1.16	1.35	1.30	0.26	4.07	1.06

hexose contents that they reported were similar to the values observed in this study. Earlier, Thompson and Brunner (1959) studied the carbohydrate content of glycoproteins indigenous to bovine milk and reported a total carbohydrate content of 6.75% for P-P. This value is significantly lower than that obtained here. Kanno and Yamauchi (1979) also observed a lower P-P carbohydrate content of 6.32%. However, Bezkorovainy et al. (1976) reported a carbohydrate composition for P-P which was quite similar to values determined in this study. They reported values of 5.1% hexose, 3.2% hexosamine, 2.0% sialic acid, and 0.5% fucose, for a total carbohydrate content of 10.8%.

Evidence derived from the immunological studies indicated that the P-P glycoprotein fraction collected from the Con A-Sepharose column was equivalent to the P-P component 3 fraction. Subsequently, discontinuous polyacrylamide gel electrophoretic results will be presented which verify that the P-P glycoprotein fraction was composed principally of component 3. This conclusion is further supported by comparing the carbohydrate compositions of the P-P glycoprotein fraction, as reported in this study, and P-P component 3, as reported by Ng et al. (1970). The present study revealed a total carbohydrate content of 18.71% for the P-P glycoprotein fraction; including 6.79% hexose, 7.12% hexosamine, 3.97% sialic acid, and 0.83% fucose. These values compare favorably with the 17.3% total carbohydrate (7.2% hexose, 6.0% hexosamine, 3.0% sialic acid, 1.1% fucose) reported for component 3 by Ng et al. (1970). The 0.59% phosphorus content observed in this study for the P-P glycoprotein fraction also agrees well with the value reported for component 3 (0.5%) by Ng (1967).

The P-P non-adsorbed fraction consists of components 5 and 8. This will be verified in the subsequent presentation of discontinuous polyacrylamide electrophoretic results. Data presented in Table 3 indicate that the P-P non-adsorbed fraction is lower in carbohydrate than the P-P glycoprotein fraction, containing only 5.62% total carbohydrate. The non-adsorbed fraction is higher in phosphorus, 1.07%, which was expected since components 5 and 8 consist of proteolytic breakdown products of β-casein, a phosphoprotein.

The s-MFGM contained 11.99% nitrogen and 9.40% total carbohydrate. These values are slightly higher than the 11.10% nitrogen and 6.48% carbohydrate reported by Brunner's group (Herald and Brunner, 1957; Thompson and Brunner, 1959).

The s-MFGM glycoprotein fraction 1 was shown by immunological techniques to contain at least one protein antigenically common to the P-P glycoprotein fraction. Data in Table 3 indicate that s-MFGM glycoprotein fraction 1 is composed of 10.00% nitrogen and 14.73% total carbohydrate. The s-MFGM glycoprotein fraction 2, in comparison, contains only 8.76% carbohydrate. Glycoprotein fraction 2 apparently bound more tightly to the Con A-Sepharose than glycoprotein fraction 1 since a higher concentration of competitive inhibitor (20% mannoside) was required for elution. Thus, one might expect glycoprotein fraction 2 to contain more carbohydrate than fraction 1. Obviously, this is not the case, therefore the difference in binding affinity between glycoprotein fractions 1 and 2 must be due to the type or availability of carbohydrate residues.

Table 4 presents the amino acid compositions of the P-P glycoprotein fraction and s-MFGM glycoprotein fraction 1. It should be recalled that

Table 4. Amino acid composition of the P-P glycoprotein fraction, the s-MFGM glycoprotein fraction 1, and P-P component 3[a].

Amino Acid	P-P glycoprotein fraction	s-MFGM glycoprotein fraction 1	P-P component 3[a]
	mole %		
Lysine	5.55	4.17	8.47
Histidine	2.38	2.31	3.53
Arginine	2.54	2.93	3.84
Aspartic acid	9.38	10.26	8.33
Threonine	10.57	9.18	7.80
Serine	12.94	8.91	9.63
Glutamic acid	15.25	10.84	16.59
Proline	5.24	5.28	7.33
Glycine	3.21	8.47	2.78
Alanine	4.68	6.79	4.42
Half Cystine	0.64	2.49	Trace
Valine	4.41	5.33	3.24
Methionine	1.80	1.27	1.81
Isoleucine	6.35	4.78	6.33
Leucine	11.32	8.48	10.69
Tyrosine	1.09	2.59	0.62
Phenylalanine	2.38	4.19	2.30
Tryptophan	0.29	1.72	Trace
	100.02	99.99	97.71

[a]Ng (1967).

these two fractions contain at least one antigenically common component, as revealed by the immunological study. The amino acid composition of P-P component 3 (Ng, 1967) is also included in Table 4. The data indicate that the amino acid composition of the P-P glycoprotein fraction compares favorably with the composition of component 3. Both preparations contain low concentrations of the aromatic amino acids, tyrosine, phenylalanine, and tryptophan, as well as low contents of the sulfur-containing amino acids, half-cystine and methionine. In addition, both preparations are high in glutamic acid, serine, and leucine. The amino acid composition of s-MFGM glycoprotein fraction 1 is substantially different from the composition of the P-P glycoprotein fraction, as indicated by the data in Table 4. This was expected because, although the glycoprotein fractions from P-P and s-MFGM contain a common protein component, their overall protein composition is different.

The amino acid chromatograms from 24 h hydrolysates of the P-P glycoprotein fraction and the s-MFGM glycoprotein fraction 1 revealed qualitative evidence that their hexosamine contents consisted of galactosamine and glucosamine.

Physical Properties

Flat-Bed Gel Electrophoresis

Flat-bed gel electrophoresis was performed to separate P-P enriched fractions into the principal components (3, 5, and 8). The components were excised and eluted from the flat-bed gel, and subjected to discontinuous polyacrylamide gel electrophoresis. The electropherograms for these components were compared to the electrophoretic pattern for

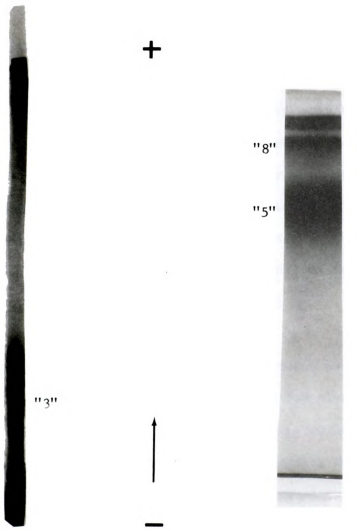


Figure 10 Flat-bed polyacrylamide gel electrophoresis of enriched proteose-peptone component 3 and enriched proteose-peptone component 5.

whole P-P, thus, each band in the disc-PAGE pattern of P-P was assigned to one or more of the classical components, viz., component 3, 5, and/or 8.

Figure 10 represents gel slices cut out of two different flat-bed gels and stained for protein with Amido Black. In one case the protein subjected to flat-bed electrophoresis was an enriched component 3 fraction, and in the other case it was enriched component 5. The bands which correspond to the individual components are apparent and identified. Cross-sectional slices, corresponding to the location of the three components, were excised from the remaining unstained gel. The protein components were eluted from the gel slices as described in the Experimental section and subjected to disc-PAGE.

Disc-PAGE

Disc-PAGE was performed using the following proteins as samples: P-P, component 3, component 5, component 8, and the P-P glycoprotein and P-P non-adsorbed fractions collected from the affinity column. Figure 11 represents photographs and Figure 12 the corresponding facsimiles of the electroporetic patterns obtained for the above samples. The separation gels contained 7.5% total acrylamide (%T) and 5.0% bisacrylamide (%C). The spacer gels consisted of 5.0% T and 20.0% C.

Plate A in Figures 11 and 12 represents the discontinuous electrophoretic patterns for P-P (A-1), component 3 (A-2), component 5 (A-3), and component 8 (A-4). A comparison of these four electropherograms permits one to assign each band in the P-P pattern to one or more of the classical components, viz., component 3, 5, and/or 8.

Plate B in Figures 11 and 12 shows the discontinuous electrophoretic patterns for P-P (B-1, B-2), the P-P glycoprotein fraction (B-3, B-4), and the P-P non-adsorbed fraction (B-5, B-6). Gels 1, 3, and 5 were stained for protein, whereas gels 2, 4, and 6 were stained for carbohydrate. The carbohydrate stained P-P gel (B-2) revealed a broad slow-moving band corresponding to component 3 and a single band in the region of component 8. It was not surprising that the broad band attributable to component 3 stained for carbohydrate, in view of its high content of carbohydrate (17.3%) reported by Ng et al. (1970). Electropherograms B-3 and B-4 represent the electrophoretic pattern of the P-P glycoprotein fraction. A comparison of patterns B-3 and A-2 reveals the similarity in electrophoretic pattern between the P-P glycoprotein fraction and P-P component 3. This electrophoretic evidence, along with the similarity in carbohydrate and amino acid compositions, enhances the conclusion that the P-P glycoprotein fraction collected from the Con A-Sepharose column is composed primarily of component 3.

The electrophoretic pattern for the P-P non-adsorbed fraction is represented by electropherograms B-5 and B-6 in Figures 11 and 12. A comparison of electropherogram B-5 with A-3 and A-4 leads to the conclusion that the non-adsorbed fraction consists principally of components 5 and 8.

Disc-PAGE was not an effective method for resolving the proteins of s-MFGM because of the formation of molecular complexes which can not enter the separation gel.

Figure 11. Disc-PAGE (7.5% T) patterns of P-P (A-1, B-1, B-2), component 3 (A-2), component 5 (A-3), component 8 (A-4), P-P glycoprotein fraction (B-3, B-4), and P-P non-adsorbed fraction (B-5, B-6). All gels were stained for protein with Coomassie Blue G-250 except gels B-2, B-4, and B-6 which were stained for carbohydrate.

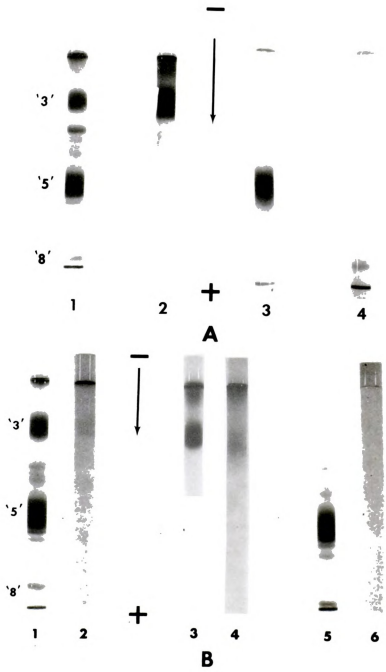
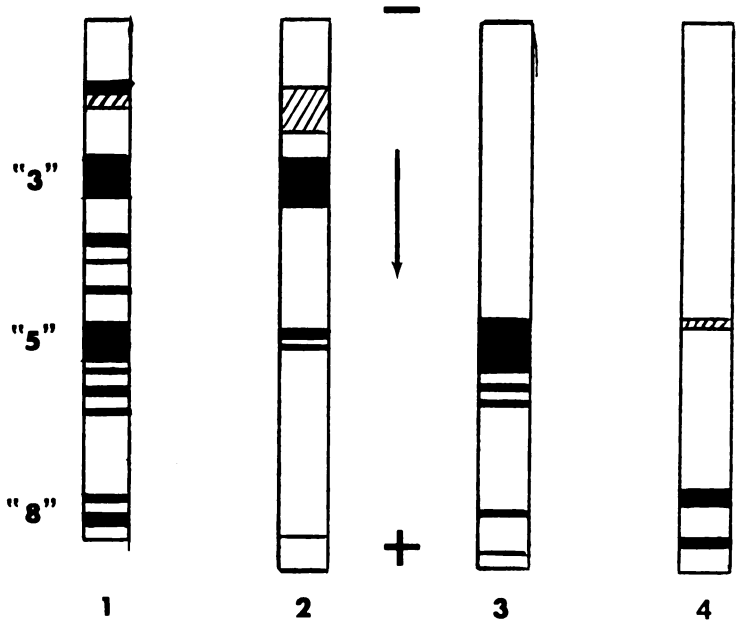
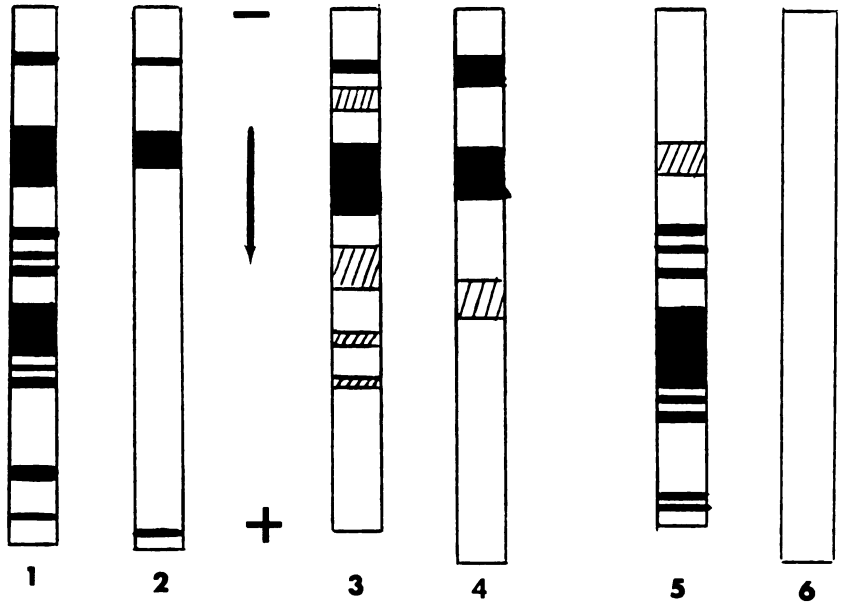


Figure 11

Figure 12. Diagrams of the electrophoretic patterns shown in Figure 11. P-P (A-1, B-1, B-2), component 3 (A-2), component 5 (A-3), component 8 (A-4), P-P glycoprotein fraction (B-3, B-4), P-P non-adsorbed fraction (B-5, B-6). All gels were stained for protein except gels B-2, B-4, and B-6 which were stained for carbohydrate.



A



B

Figure 12

SDS-PAGE

Polyacrylamide gel electrophoresis in the presence of SDS was performed with P-P, s-MFGM, and the fractions obtained from the Con A-Sepharose column.

Photographs and facsimiles of the SDS-PAGE patterns developed for P-P and its Con A-Sepharose derived fractions are presented in Figure 13. The series A gels were stained for protein, whereas the series B gels were stained for carbohydrate. Of particular interest is the SDS-PAGE pattern of the P-P glycoprotein fraction which was demonstrated by previous chemical and electrophoretic techniques to consist principally of component 3. The SDS-PAGE pattern of this fraction (A-3) showed two major bands, one with a molecular weight ranging from 17,700 to 20,900, and a broader band of approximately 24,600 to 33,400 daltons, as well as three minor higher molecular weight zones. All of these protein zones, except one of the minor zones, stained for carbohydrate (B-2). This characteristic was anticipated since the protein components of this fraction interacted with Con A. It is interesting to note that the SDS-PAGE pattern of the P-P non-adsorbed fraction (A-4) also stained for carbohydrate to a limited extent (see pattern B-3). Apparently, there exists a small quantity of glycoprotein in P-P which does not exhibit binding affinity for Con A.

Figure 14 represents the SDS-PAGE electropherograms of s-MFGM and its Con A-Sepharose derived fractions. The immunological analyses previously discussed revealed that s-MFGM glycoprotein fraction 1 contained at least one glycoprotein which was antigenically similar to a component in the P-P glycoprotein fraction. In an effort to identify

Figure 13. SDS-PAGE (10% T, 2.63% C) patterns and diagrams of P-P (A-2, B-1), P-P glycoprotein fraction (A-3, B-2), and P-P non-adsorbed fraction (A-4, B-3). The series A gels were stained for protein with Coomassie Blue R-250 and the series B gels were stained for carbohydrate. Gel A-1 is a standard protein mixture with molecular weights in units of one thousand.

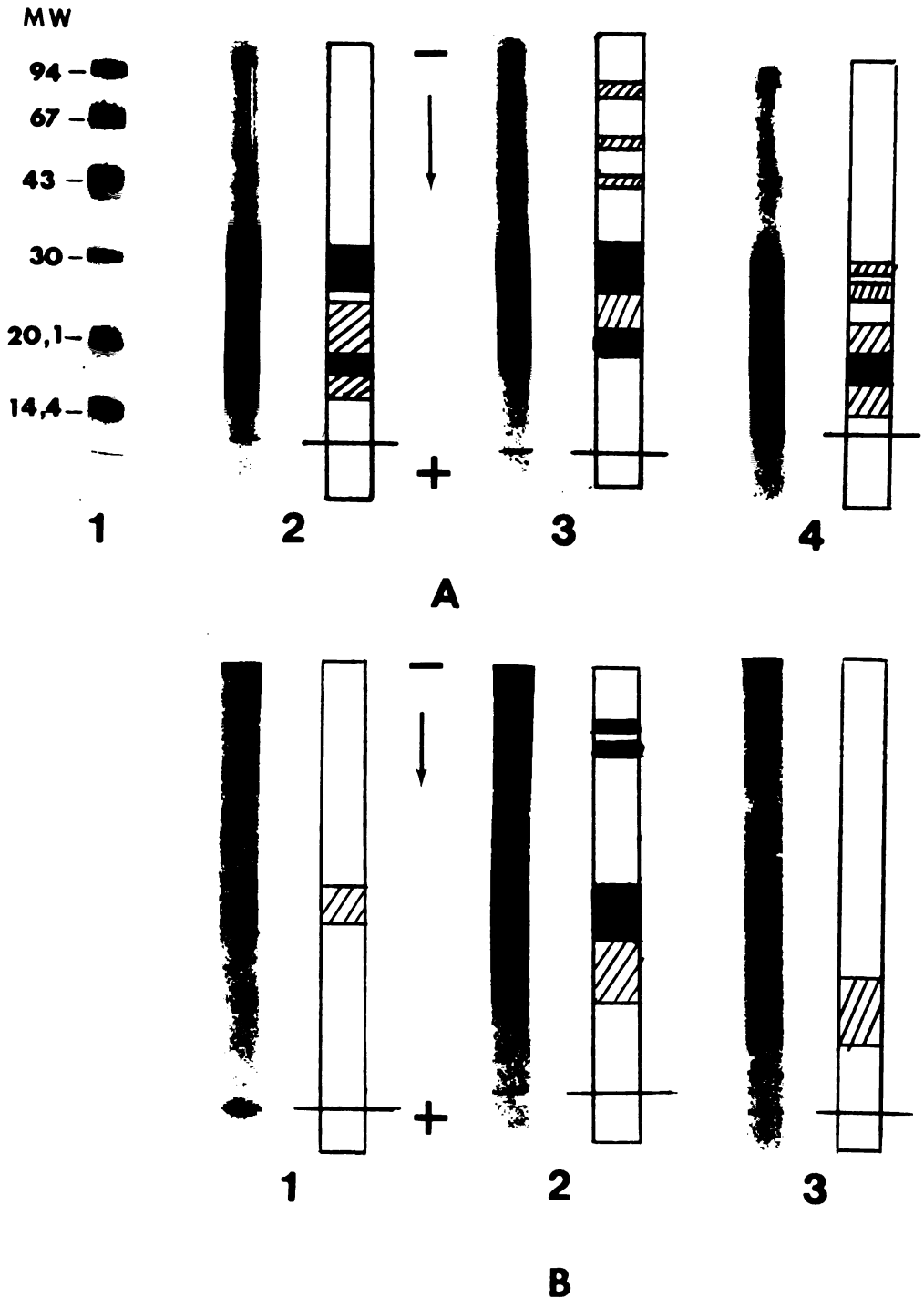


Figure 13

Figure 14. SDS-PAGE (10% T, 2.63% C) patterns and diagrams of s-MFGM (A-2, B-1), s-MFGM glycoprotein fraction 1 (A-3, B-2), and s-MFGM non-adsorbed fraction (A-4, B-3). The series A gels were stained for protein with Coomassie Blue R-250 and the series B gels were stained for carbohydrate. Gel A-1 is a standard protein mixture with molecular weights in units of one thousand.

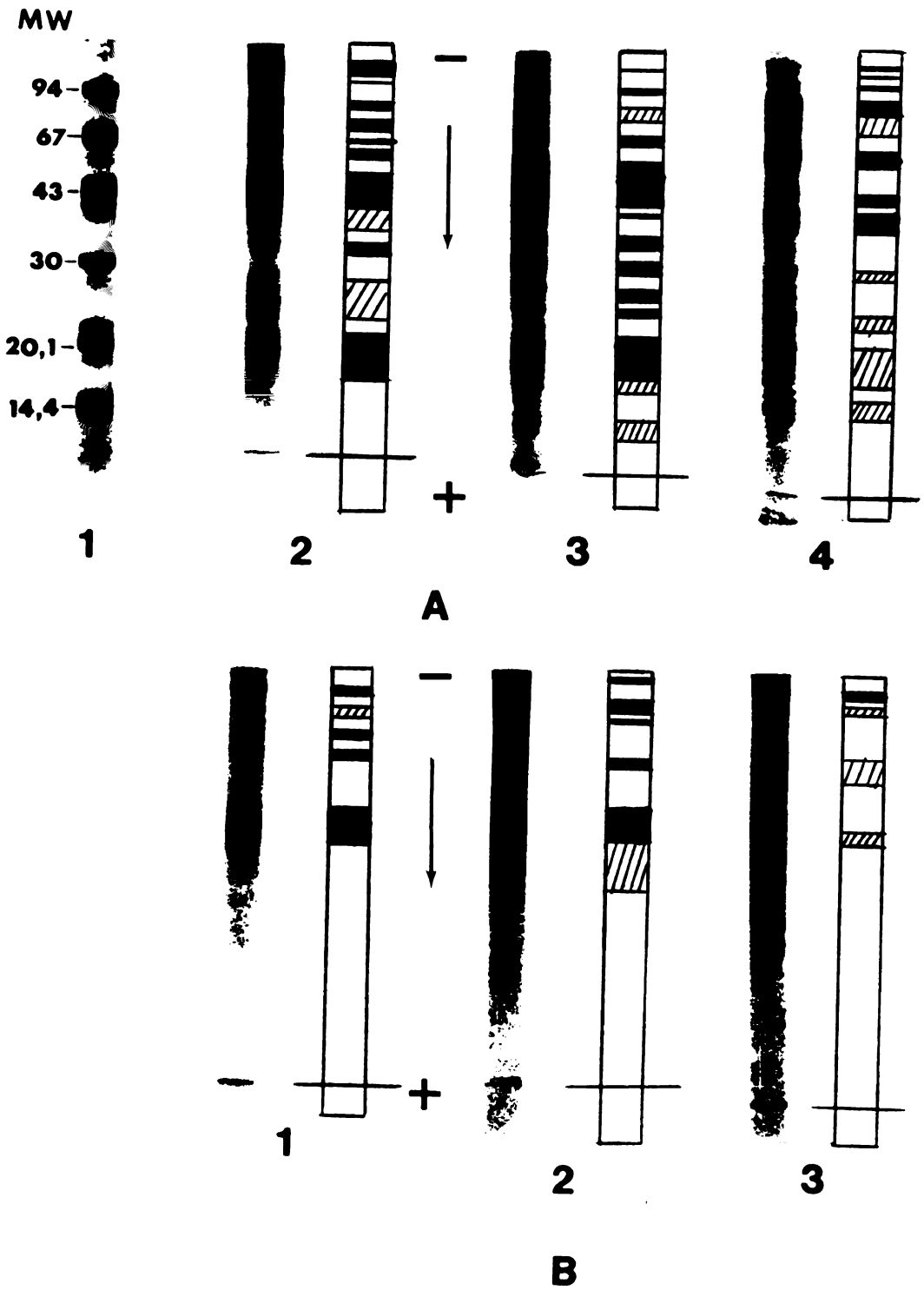


Figure 14

the antigenically common protein(s) in these two glycoprotein fractions, their SDS-PAGE patterns were compared; see the comparative facsimiles in Figure 15. Pattern A is the P-P glycoprotein fraction and pattern B is the s-MFGM glycoprotein fraction 1. As indicated, there are several protein zones which migrated similarly in both gels and are presumed to be of approximately equal molecular weights. The major zone common to both patterns exhibited a molecular weight of approximately 18-21,000 (see Figure 15). In addition, there were three minor bands in the SDS-PAGE pattern of s-MFGM glycoprotein fraction 1 (B) which appeared to overlap the broad 24,600-33,400 molecular weight area in the pattern of the P-P glycoprotein fraction (A). These three bands exhibited molecular weights of 24,800, 28,200, and 32,200. The interpretation was made that one or more of these protein bands, viz., 18-21,000, 24,800, 28,200, or 32,200, was the species from s-MFGM which is immunologically reactive with anti-proteose-peptone. To ascertain which of these common molecular weight species was the anti-proteose-peptone reactive component, the experiment outlined in the next section was performed.

Before proceeding to the next section, Figure 14 reveals two additional characteristics which should be noted. First, the carbohydrate stained pattern of s-MFGM glycoprotein fraction 1 (B-2) did not reveal all of the bands observed in gels stained for protein (A-3). This was an interesting observation because of the glycoprotein nature of this fraction which bound to Con A. Perhaps the concentration of the unstained bands was too low to be detected by the carbohydrate staining procedure used. Secondly, the s-MFGM non-adsorbed fraction showed several bands which stained for carbohydrate (see pattern B-3). These

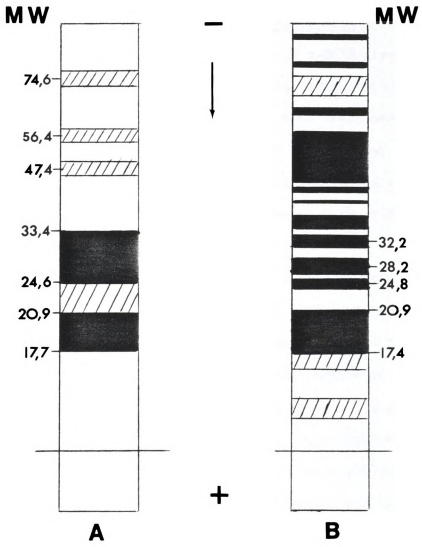


Figure 15. Comparison of the SDS-PAGE (10% T, 2.63% C) patterns of P-P glycoprotein fraction (A) and s-MFGM glycoprotein fraction 1 (B). Molecular weights, in units of one thousand, are shown for selected bands.

glycoproteins either contain carbohydrate residues for which Con A does not show binding specificity, or in some manner sterically hinder their interaction with Con A.

Identification of an Anti-Proteose-Peptone Reactive Species From s-MFGM

A comparison of the SDS-PAGE patterns of s-MFGM glycoprotein fraction 1 and the P-P glycoprotein fraction (Figure 15) revealed four protein zones which migrated similarly in both fractions, yielding equal molecular weights, viz., 18-21,000, 24,800, 28,200, and 32,200 daltons. At least one of these common molecular weight proteins is probably responsible for the antigenic similarity between the P-P glycoprotein fraction and s-MFGM glycoprotein fraction 1.

To ascertain which protein bands were reacting with anti-proteose-peptone, i.e., which protein components are antigenically common to both P-P and s-MFGM, the experiment outlined in Table 5 was performed. Eighteen SDS-PAGE gels were electrophoresed using s-MFGM glycoprotein fraction 1 as the sample in each gel. Following electrophoresis, the gels were sliced into twenty-four segments, each 2.3 mm thick. Common segments from each of the eighteen gels were combined, macerated in 1.0 ml of 1% Triton X-100, and incubated overnight with periodic mixing to ensure the complete diffusion of protein from the gel fragments. Treatment of the gel segments with Triton X-100 was performed to negate the possible interference of adsorbed SDS on the subsequent immuno reactions (Dimitriadis, 1979; Russell, 1979). Finally, immuno-double diffusion experiments were performed with the protein extracted from each gel segment, i.e., the antigens, against anti-proteose-peptone

Table 5. Protocol for identification of an anti-proteose-peptone reactive component in s-MFGM glycoprotein fraction 1.

1.	SDS-PAGE (10% T, 2.63% C) was performed with s-MFGM glycoprotein fraction 1 as the sample. Eighteen gels were run.
2.	The gel cylinders were sliced into 2.3 mm thick slices (twenty-four total slices), and the common slices from each of the gels were combined (for example, the first slice from all eighteen gels were combined, etc.).
3.	The gel slices were macerated in 1.0 ml of 1% Triton X-100 and incubated overnight with periodic mixing.
4.	Immuno-double diffusion was performed with the protein eluted from each gel slice reacted against anti-proteose-peptone.

immune serum. The objective of this experiment was to identify the anti-proteose-peptone reactive species in the SDS-PAGE pattern of s-MFGM glycoprotein fraction 1.

Figure 16 shows a facsimile of the SDS-PAGE pattern of s-MFGM glycoprotein fraction 1 (A), as well as a representation of the template pattern employed for the immuno-double diffusion experiments (B). The protein extracted from each gel segment was applied to three antigen wells as illustrated in Figure 16 for gel segments 1 and 2. The antigen wells were 5 mm in diameter, and were spaced 3, 6, and 9 mm, respectively, from the antibody trough. Multiple antigen wells at varying distances from the antibody trough were employed to ensure antigen-antibody equivalence. After filling the antibody trough with anti-proteose-peptose immune serum, the plates were allowed to stand in a humid environment at room temperature for several days.

The results of these immuno-double diffusion experiments are presented in Figure 17. Photographs of six plates are shown, with the antigen wells numbered to correspond with the specific gel segments. Protein extracted from gel segments 1-16 and 21-24, inclusive, did not react with anti-proteose-peptone. However, broad precipitin bands developed with the protein extracted from gel segments 17, 18, 19, and 20. These precipitin bands indicate that the protein extracted from segments 17-20, inclusive, is antigenically common to both P-P and s-MFGM. The molecular weight range corresponding to segments 17-20, inclusive, is approximately 17,000-22,500. Therefore, the common protein zone of 18-21,000 molecular weight, observed in the SDS-PAGE patterns of s-MFGM glycoprotein fraction 1 and the P-P glycoprotein

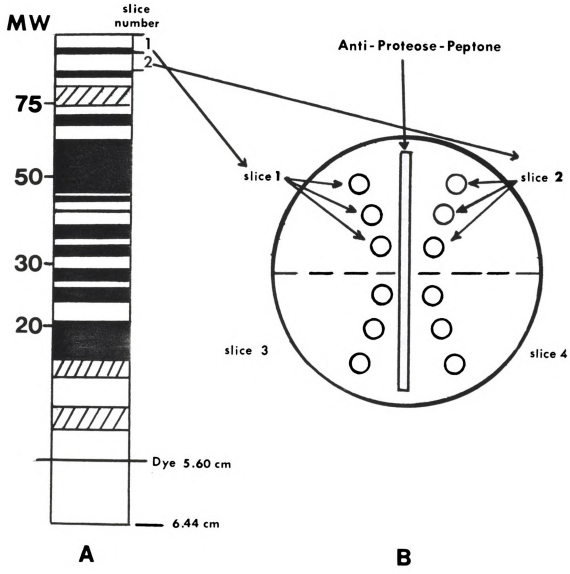
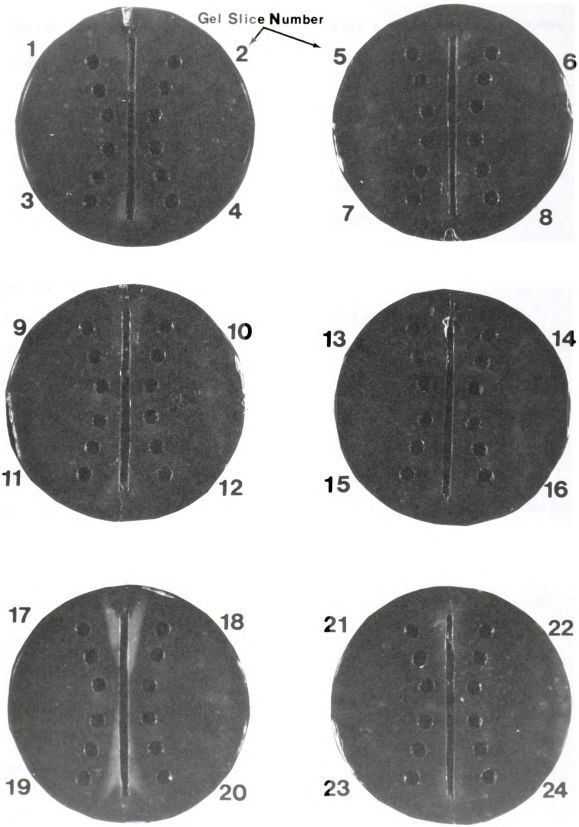


Figure 16. A. SDS-PAGE (10% T, 2.63% C) pattern of s-MFGM glycoprotein fraction 1. Following electrophoresis the unstained gel was sliced into twenty-four 2.3 mm thick slices (see Table 5). The molecular weight range shown to the left of the diagram is in units of one thousand.

B. Representation of the template pattern for immuno-double diffusion of the protein eluted from each gel slice against anti-proteose-peptone. The protein eluted from each slice was applied to three antigen wells located 3, 6, and 9 mm, respectively, from the antibody trough.

Figure 17. Photographs of immuno-double diffusion experiments involving reaction of anti-proteose-peptone against the protein eluted from each slice of SDS-PAGE gel. The protein subjected to SDS-PAGE was s-MFGM glycoprotein fraction 1. Note the precipitin bands which developed with the protein eluted from gel slices 17, 18, 19 and 20.


Gel Slice Number
1
2
3
4
5
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17
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23
24

Figure 17

fraction (see Figure 15), is the antigenically similar component in the two glycoprotein fractions. The fact that the precipitin bands observed in Figure 17, representing gel segments 17-20, are rather broad and diffuse suggests some denaturation of the protein by residual SDS. Though Triton X-100 aids in counteracting the inhibition due to SDS, it probably does not fully restore the antigenicity of the protein.

The conclusion derived from this experiment is that there is a glycoprotein of molecular weight 18-21,000 antigenically common to both the P-P component 3 fraction and s-MFGM, thereby suggesting that the fat globule membrane is the origin for this P-P glycoprotein. It also appears that there are additional glycoproteins in P-P component 3 which are not derived from the fat globule membrane. For example, the broad 24,600-33,400 zone, as well as the three higher molecular weight bands observed in the SDS-PAGE pattern of the P-P glycoprotein fraction (see Figure 15), do not appear to originate from the fat globule membrane. These glycoproteins may possibly originate from the blood of the cow or represent degradation products of a parent glycoprotein.

The glycoprotein in P-P which appears to be derived from the fat globule membrane, i.e., the 18-21,000 molecular weight component, may originate as a result of biologically or physically induced membrane degradation. Biologically induced degradation refers to proteolysis of the membrane proteins, whereas, physically induced degradation refers to the physical sloughing of protein which may occur during secretion of the fat globule or as a result of mishandling of the milk.

Research directed toward the characterization of the P-P components, as well as a determination of their specific origins, may eventually reveal their significance in milk. Other than the observation that components of P-P account for the relatively low surface tension of milk, the significance of these components to the properties of milk and milk products remains to be determined. The possibility exists that the concentration of certain P-P components may reflect the health of the cow, age of the milk, and/or the manner in which the milk was handled or processed.

CONCLUSIONS

Immunological techniques demonstrated the presence of at least one antigenically similar component in proteose-peptone (P-P) and the soluble protein of the fat globule membrane (s-MFGM).

Affinity chromatography with a Con A-Sepharose support was used to fractionate P-P and s-MFGM. Proteose-peptone was fractionated into a P-P non-adsorbed fraction, accounting for 66% of the protein, and a P-P glycoprotein fraction which accounted for the remainder of the protein. The s-MFGM was fractionated into three fractions; a s-MFGM non-adsorbed fraction which accounted for 21% of the protein, a s-MFGM glycoprotein fraction 1 (eluted with 5% mannoside) which contained 48% of the protein, and a s-MFGM glycoprotein fraction 2 (eluted with 20% mannoside) which accounted for 10% of the protein. The remaining 20% of s-MFGM bound to the Con A-Sepharose and was not eluted.

The P-P glycoprotein fraction was shown by carbohydrate determination (18.71% total carbohydrate), phosphorus determination (0.59% P), amino acid analysis, and disc-PAGE to consist primarily of the component 3 fraction. The P-P non-adsorbed fraction consisted principally of components 5 and 8.

Of the fractions separated from s-MFGM by affinity chromatography, only the s-MFGM glycoprotein fraction 1 was immunologically reactive with anti-proteose-peptone, exhibiting an arc of identity with the P-P glycoprotein fraction. This indicated that these two glycoprotein fractions contained at least one antigenically similar component.

Electrophoretic comparison (SDS-PAGE) of the P-P glycoprotein fraction with s-MFGM glycoprotein fraction 1 revealed four protein zones common to both fractions, with apparent molecular weights of 18-21,000, 24,800, 28,200, and 32,200. Immuno-double diffusion experiments identified the 18-21,000 molecular weight species as the antigenically common component in the two glycoprotein fractions.

The evidence presented suggests that s-MFGM is the probable origin for an 18-21,000 molecular weight glycoprotein in the P-P component 3 fraction. There are additional glycoproteins in P-P which apparently are not derived from s-MFGM. These glycoproteins may originate from the blood of the cow or represent degraded fractions of a parent glycoprotein.

APPENDICES

APPENDIX A

Interaction of Con A with glucose and mannose residues

Con A is a metalloprotein and a lectin which is isolated from the jack bean (Canavalia ensiformis). Lectins are proteins which can act as agglutinating agents. They have specific affinities for various sugar residues and can bind to these sugar residues to form a complex in much the same manner that an enzyme binds to a substrate or an antibody to a specific antigen (Sharon, Resiner, Ravid, and Prujanksy, 1979). Although the lectin-saccharide interaction has been compared to antigen-antibody reactions, lectins are not of immune origin and do not display as high a degree of specificity as antibodies do. The lectin-saccharide complex formation is, however, a specific reaction and for a certain lectin to bind to a polysaccharide or glycoprotein, that macromolecule must contain sugar residues for which the lectin shows specificity. Furthermore, the specific sugar residues must be located in positions on the carbohydrate chain which facilitate interaction with the saccharide binding site on the lectin molecule. For many lectins the specific sugar residues must be in the terminal position of the non-reducing end of the carbohydrate chain (Brewer and Brown, 1979).

Lectins may show specificity for only a single sugar residue, or they may have a broader specificity, reacting with two or more structurally related sugars. Con A exhibits binding specificity for α-D-glucopyranosyl and α-D-mannopyranosyl residues as well as weaker binding affinity for certain derivatives of these sugars (Goldstein, Hollerman, and Smith, 1965). Examination of the structures of α-D-glucose and α-D-mannose reveals that both sugars have the D-arabino configuration at carbon atoms 3, 4, 5, and 6. The D-arabino configuration at these carbon atoms is required for complex formation between a sugar residue and Con A (Goldstein et al., 1965). The binding mechanism between Con A and either mannose or glucose involves hydrophobic bonding, as well as the formation of hydrogen bonds between the hydroxyl groups at carbons 3, 4, and 6 of the sugar and the carboxylic acid side chains and carbonyl oxygens of the amino acids near the Con A binding site. Unmodified hydroxyl groups at the 3, 4, and 6 carbon atoms are essential for the binding of mannose or glucose to Con A.

Con A will bind to any glycoprotein containing α-D-mannose or α-D-glucose residues with unmodified hydroxyl groups at carbons 3, 4, and 6. The sugar residues may be located either at the non-reducing terminal end of a carbohydrate chain or in an internal position linked by an α-1, 2 glycosidic bond. In both cases the three hydroxyl groups required for hydrogen bond formation with Con A have remained unmodified. Con A has been reported to have a relatively strong affinity for the mannobiosyl-N-acetylglucosamine sequence which is present in many glycoproteins (Dulvaney, 1979).

APPENDIX B

Staining Procedures

Protein

A. Coomassie Blue R-250 (Weber and Osborn, 1969)

The staining solution was prepared by dissolving 1.25 g of Coomassie Blue R-250 in a mixture of 454 ml of 50% methanol and 46 ml of glacial acetic acid. The solution was filtered through Whatman No. 1 filter paper prior to use. Polyacrylamide gel cylinders were immersed in the stain for 2-10 h at room temperature, rinsed with distilled water, and destained in a diffusion destainer containing 75 ml glacial acetic acid plus 50 ml of methanol and 875 ml distilled water. The gel cylinders were stored in 7% acetic acid after destaining.

B. Coomassie Blue G-250 (Reisner, Nemes, and Buchalty, 1975).

The staining solution was prepared by diluting 400 mg of Coomassie Blue G-250 and 50 g of 70% perchloric acid to 1.0 liter with distilled water. This gives a 0.04% G-250 in 3.5% perchloric acid solution. The stain was stirred overnight and filtered through Whatman No. 1 filter paper before using. Gel cylinders were immersed in the staining solution overnight and then destained in 7% acetic acid in the diffusion destainer. Gels were stored in 7% acetic acid.

Carbohydrate

The staining procedure of Zacharius, Zell, Morrison, and Woodlock (1969) was used to visualize glycoproteins in the electrophoretic gel patterns from both Disc-PAGE and SDS-PAGE. The fuchsin-sulfite stain was prepared as follows. To 2.0 liters of distilled water was added 16.0 g of potassium metabisulfite and 21.0 ml of concentrated HCl. Eight grams of finely powdered basic fuchsin was added and the mixture stirred for 2 h at room termperature. After allowing the dye solution to stand for an additional 2 h, a small amount of decolorizing charcoal was added, stirred for 5 min, and the solution filtered under vacuum through Whatman No. 1 filter paper. This last step was repeated until a colorless solution was obtained, which was stored at $4^{\circ}C$.

After electrophoresis gel cylinders were immersed in 12.5% TCA for 30 min to fix the proteins. The gels were then rinsed lightly with distilled water and immersed in a solution of 1% periodic acid in 3% acetic acid for 50 min. Washing of each gel in 200 ml of distilled water was carried out overnight. It is essential to completely remove the periodic acid in the washing step in order to obtain a stained gel with a clean background. After washing, the gels were soaked in the fuchsin-sulfite stain for 50 min in the dark, followed by three 10 min washes with freshly prepared 0.5% metabisulfite solution. Glycoproteins were stained red. Background staining was removed by soaking the gels in a diffusion destainer containing distilled water. Gels were stored in 7% acetic acid.

It has been reported that SDS can bind dye and thus contribute to heavy background staining (Fairbanks, Steck, and Wallach, 1971).

Therefore it was desirable to remove SDS from SDS-PAGE gels prior to staining for glycoproteins. After soaking the gel cylinders in 12.5% TCA for 30 min, the gels were immersed overnight in a mixture of 25% isopropyl alcohol and 10% acetic acid. They were then transferred to 10% ispropyl alcohol-10% acetic acid for 6-9 h, followed by soaking in 10% acetic acid overnight. After rinsing with distilled water the gels were stained for carbohydrate.

Table A1. List of chemicals used in this study.

Chemical	Company
Ammonium persulfate	Baker
Selenium dioxide	"
Sodium carbonate	"
Acrylamide	Bio-Rad
Bisacrylamide	"
Sodium dodecyl sulfate	"
N-Acetyl neuraminic acid	Calbiochem
Fucose	"
Galactose	"
Mannose	"
Pronase	"
Agar	Difco
Freund's adjuvant	"
Basic fuchsin	Eastman Kodak
Photo-Flo 200	"
N, N, N^1, N^1-tetramethylethylenediamine	"
Thiobarbituric acid	"
Acetylacetone	Fisher
Ammonium sulfate	"
Boric acid	"
Bromophenol blue	"
Phosphoric acid	"
Potassium metabisulfite	"
Potassium phosphate, monobasic	"
Sodium phosphate, monobasic	"
Sodium sulfate	"
Acetic acid	Mallinckrodt
Ammonium molybdate	"
Barbital	"
Citric acid	"
Cupric sulfate	"
Cyclohexanone	"
Diethyl ether	"
Hydrochloric acid	"
Hydrogen peroxide	"
Isopropyl alcohol	"
2-mercaptoethanol	"
Methanol	"
Methyl red	"
Perchloric acid	"
Phenol	"
Phosphorus pentoxide	"
Potassium sodium tartrate	"
Sodium arsenite	"

Table A1.--Continued

Chemical	Company
Sodium bicarbonate	Mallinckrodt
Sodium chloride	"
Sodium hydroxide	"
Sodium nitrite	"
Sucrose	"
Sulfuric acid	"
Trichloroacetic acid	"
Bromocresol green	Matheson, Coleman and Bell
p-Dimethylaminobenzaldehyde	"
Ferrous sulfate	"
Sodium meta-periodate	"
Sodium phosphate, dibasic	"
Decolorizing charcoal	Pfanstiehl Chemicals
Low MW protein calibration kit	Pharmacia
Bovine serum albumin	Sigma
Coomassie Brilliant Blue G-250	"
Coomassie Brilliant Blue R-250	"
Cysteine-HCl	"
Galactosamine-HCl	"
Glucosamine-HCl	"
α-Methyl-D-mannoside	"
Sodium azide	"
Tris (trishydroxymethylaminomethane)	"
Tryptophan	"
Glycine	U.S. Biochemical Corp.

Table A2. Preparation of solutions used in various experiments.

Experiment		
Hexosamine	0.5 N	Sodium carbonate-sodium bicarbonate, pH 9.8, 0.1 M NaCl: 23.02 g sodium carbonate + 2.76 g sodium bicarbonate + 5.84 g NaCl made to 1.0 liter with distilled water.
Sialic Acid	0.2 M	Sodium meta-periodate in 9 M phosphoric acid: 0.428 g sodium periodate + 6.04 ml concentrated H_3PO_4 made to 10.0 ml with distilled water.
	10%	Sodium arsenite in 0.5 M sodium sulfate-0.1 N H_2SO_4: 1.7756 g sodium sulfate + 2.50 g sodium arsenite made to 25.0 ml with 0.1 N H_2SO_4.
	0.6%	Thiobarbituric acid (TBA) in 0.5 M sodium sulfate: 0.600 g TBA + 7.1024 g sodium sulfate made to 100 ml with distilled water.
Tryptophan	0.1 M	Sodium phosphate, pH 7.5: 0.222 g $NaH_2PO_4 \cdot H_2O$ + 2.253 g $Na_2HPO_4 \cdot 7H_2O$ made to 200 ml with distilled water.
Disc-PAGE	0.380 M	Tris-HCl, pH 8.9: 23.009 g Tris + water + HCl to pH 8.9 made to 500 ml with distilled water.
	0.062 M	Tris-HCl, pH 6.7: 1.877 g Tris + water + HCl to pH 6.7 made to 250 ml with distilled water.
	0.046 M	Tris-glycine, pH 8.3: 11.132 g Tris + 57.6 g glycine made to 2000 ml with distilled water.

Table A2.--Continued

Experiment		
SDS-PAGE	0.1 M	Sodium phosphate, pH 7.2, 0.1% SDS: 1.95 g $NaH_2PO_4 \cdot H_2O$ + 9.65 g $Na_2HPO_4 \cdot 7H_2O$ + 0.5 g SDS made to 500 ml with distilled water.
	0.01 M	Sodium phosphate, pH 7.0, 3% SDS, 1% 2-mercaptoethanol: 0.138 g $NaH_2PO_4 \cdot H_2O$ + 3.0 g SDS + 1.0 ml 2-mercaptoethanol made to 100 ml with distilled water.
Immuno-double diffusion		Veronal buffer, pH 8.6, ionic strength = 0.05: 1.26 g barbital + 3.8 g sodium acetate + 9.74 g sodium barbital made to 2.0 liters with distilled water.
		Phosphate buffered saline, pH 7.0: 12.25 g KH_2PO_4 + 2.05 g NaOH + 17.55 g NaCl + 0.4 g NaN_3 made to 2.0 liters with distilled water.

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