Study of a Hydrophobic Protein Fraction Isolated from Milk Proteose-Peptone

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ABSTRACT
Hydrophobic interaction chromatography was used to isolate a hydrophobic fraction from proteose-peptone. Further fractionations by means of gel permeation chromatography and SDS-PAGE led to determining four protein groups. Group 2 containing principally component 3 was especially investigated. Electrophoretic analysis resulted in two major polypeptides with apparent molecular weights of 17,000 and 7000; the largest reacting as a glycoprotein is thought to be an anti-milk fat globule membrane-reacting protein.

INTRODUCTION
Among milk proteins, the proteose-peptone fraction is the least well understood. It is commonly thought to be composed of two groups of proteins. The first group is polypeptides resulting from proteolysis of caseins (CN) by indigenous proteases in milk, among which the components 5, 8F, and 8S are well-known (2, 3, 6, 7) and were recently listed as β-CN-5P, β-CN-4P, and β-CN-1P in a report of the Committee on Nomenclature and Methodology of Milk Proteins of the Dairy Foods Research Section, Dairy Food Division, American Dairy Science Association (8). The second group comprises other polypeptides, among which component 3 (PP3) appeared to be a genuine whey protein.

Because of the inhibitory effect of proteose-peptone and especially of PP3-enriched fractions on lipolysis in milk (1, 5) it is of interest to elucidate the origin and the composition of PP3. A glycoprotein fraction shown to consist principally of PP3 has been isolated; it contains at least one antigenically similar component with the soluble protein of milk fat globule membrane (MFGM) (11). We have recently studied the immunological relationship between a hydrophobic fraction of proteose-peptone (HFPP) containing PP3 and MFGM (16). The objective of the present study was to explore the composition of this HFPP and, in particular, PP3.

MATERIALS AND METHODS

Materials
Phenyl-Sepharose CL 4 B, Sephacryl S200 Superfine, and Superose 12 HR 10/30 column were from Pharmacia Fine Chemicals (Uppsala, Sweden). Hydroxyapatite Ultrogel was from IBF Pharmindustrie (Clichy, France). Acrylamide and bis acrylamide were from BDH (Poole, England).

Extraction of Proteose-Peptone
The proteose-peptone used corresponds to the “Sigma-proteose” (4). Raw skimmed milk was heated (95°C, 30 min); after it cooled at 20°C and pH decreased to 4.6 with NHCl, it was centrifuged (5000 x g, 30 min). The supernatant was then treated with ammonium sulfate (half saturation); after centrifugation (5000 x g, 30 min) the proteose-peptone pellet was dissolved in distilled water, exhaustively dialyzed, and then lyophilized.

Hydrophobic Interaction Chromatography
The hydrophobic interaction chromatographic (HIC) procedure was derived from the one previously described (18, 19). Phenyl-Sepharose CL 4B, equilibrated in 0.01 M pH 6.8 sodium phosphate buffer containing 0.35 M ammonium sulfate, was poured into a column (2.6 x 20 cm); then the proteose-peptone extract dissolved in the same buffer was loaded
in the column. After a first washing step, at a flow rate of 38 ml/h, during which the unbound proteins were eliminated, the hydrophobic proteins were eluted with .01 M pH 6.8 sodium phosphate buffer containing ethylene glycol 50%.

Hydroxyapatite Chromatography

The slurry of hydroxyapatite-Ultrogel, previously equilibrated in .01 M pH 6.8 potassium phosphate buffer, was poured into a column (1.6 x 15 cm). The protein fraction, dialyzed against the same buffer, was loaded in the column. After the washing step, at a flow rate of 20 ml/h, the proteins, adsorbed on the hydroxyapatite, were eluted with .25 M pH 6.8 potassium phosphate buffer.

Gel Permeation Chromatography
Low Pressure Chromatography

The protein fractions dialyzed against .1 M Tris/HCl, .5 M NaCl, pH 8 buffer, were filtered through a column (2.6 x 95 cm) of Sephacryl S200 with the same buffer at a flow rate of 54 ml/h or 37 ml/h.

High Performance Liquid Chromatography

Size exclusion chromatography was utilized using a Superose 12 HR 10/30 column equilibrated with .05 M potassium phosphate, .15 M NaCl, pH 7.2 buffer. Elutions were carried out at a flow rate of .25 ml/min. Samples sizes were 30 to 50 µg of proteins in 75 µl of buffer.

Electrophoresis

Native PAGE by the method of Hillier (9) was adapted to a vertical gel slab apparatus with minor modifications, including a spacer gel (T: 3.9%; C: 2.7%) pH 6.8. The SDS-PAGE on vertical gel slabs was carried out according to Laemmli and Favre (12). Proteins were stained by Coomassie blue R 250; glycoproteins were revealed by Schiff’s reagent (10).

Compositional Analyses

Proteins were determined by the procedure of Lowry et al. (14). Amino acid analyses were performed on 24 h hydrolysates with a Technicon NC 2 P amino acid analyzer (15). Tryptophan was estimated by a spectrometric method (13). Cystine was determined following a preliminary oxidation with performic acid.

RESULTS

Hydrophobic Interaction Chromatography

The analytical method previously described (18, 19) was adapted to a semi-preparative scale with a two-step gradient (Figure 1). The first fraction eluted during the washing step contains β-CN-5P, β-CN-4P, and β-CN-1P and probably other minor proteose-peptone components.

The second fraction whose elution required the use of phosphate buffer free of ammonium sulfate, but the presence of 50% ethylene glycol, contains PP3 and some unknown components of varying electrophoretic mobilities; an opalescent appearance is noted in this last fraction. In accordance with its hydrophobic properties, we name it HFPP (Figure 2).

Hydroxyapatite Chromatography

After dialysis against .01 M pH 6.8 potassium phosphate buffer, HFPP was chromatographed on an hydroxyapatite column (Figure 3). During the washing step, substances with relatively high UV absorbance, but not stained by the Coomassie blue on the PAGE, were separated (HA1). The second fraction (HA2), eluted during the increasing ionic strength gradient, contains all the protein components of HFPP (Figure 2) and becomes absolutely clear.

Journal of Dairy Science Vol. 71, No. 6, 1988
Figure 2. Native PAGE patterns of chromatographic fractions. HFPP: Fraction 2 of Phenyl-Sepharose chromatography. HA1 and HA2: Fractions of hydroxyapatite chromatography. 1a to 4a: Fractions of gel filtration at a flow rate of 54 ml/h. T: Whole proteose-peptone (PP3) fraction. Division lines between H1 to H4 were put arbitrarily to separate groups of electrophoretic bands. CN = Casein.

Figure 3. Chromatography of the hydrophobic fraction of proteose-peptone on a column (1.6 × 15 cm) of Hydroxyapatite-Ultrogel in .01 M potassium phosphate, pH 6.8 buffer. Fraction 2 was eluted with .25 M potassium phosphate. Flow rate 20 ml/h.

Gel Permeation Chromatography

The gel permeation chromatography (GPC) of the clear HFPP on a Sephacryl S200 column at a flow rate of 54 ml/h (Figure 4) separated four fractions. The electrophoretic patterns (Figure 2) show that each fraction (1a to 4a) contains proteins with increasing electrophoretic mobilities so that we were able to distinguish four groups of bands named H1 to H4. H1 is assumed to be composed of proteins of high molecular weights, which have difficulty penetrating the gel. H2 contains principally one band presenting the characteristic mobility of PP3. H3 contains bands of in-
termediate mobility, which compose the fractions 2a and 3a. H4 comprises bands of high mobility representing the fraction 4a.

The decreasing flow rate from 54 to 37 ml/h (Figure 4b) allowed separation of Groups H1 and H2, respectively, into fractions 2b and 3b but made the fractionation of Groups H3 and H4 difficult (Figure 5).

Control of the Fractions of Gel Permeation Chromatography by Means of High Performance Liquid Chromatography and Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

The apparent molecular weights of the
TABLE 1. Estimation of the apparent molecular weight of components of gel permeation chromatographic (GPC) fractions. Under lined values correspond to predominant peaks or bands.

<table>
<thead>
<tr>
<th>GPC Fraction</th>
<th>Native-PAGE Group</th>
<th>HPLC Apparent molecular weight (Mr)</th>
<th>SDS-PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1b</td>
<td>NT</td>
<td>NT</td>
<td>70,000&gt;Mr&gt;50,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60,000</td>
</tr>
<tr>
<td>2b</td>
<td>H1</td>
<td>&gt;300,000</td>
<td>17,000&gt;Mr&gt;15,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;10,000</td>
<td>7000&gt;Mr&gt;6000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&gt;300,000 P1</td>
<td>17,000&gt;Mr&gt;15,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100,000&gt;Mr&gt;70,000</td>
<td>P2: 7000&gt;Mr&gt;6000</td>
</tr>
<tr>
<td>3b</td>
<td>H2</td>
<td></td>
<td>Many bands in the range</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;12,000</td>
<td>17,000 to 7000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>40,000</td>
</tr>
<tr>
<td>4b</td>
<td>H3 + H4</td>
<td>70,000&gt;Mr&gt;15,000</td>
<td>8000&gt;Mr&gt;7000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;12,000</td>
<td>15,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>9000</td>
</tr>
<tr>
<td>5b</td>
<td>H3 + H4</td>
<td>15,000</td>
<td>40,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>&lt;12,000</td>
<td></td>
</tr>
</tbody>
</table>

1 Not tested.

components of each GPC fraction have been estimated using HPLC filtration and SDS-PAGE; data were copied out on Table 1.

Fractions 2b to 5b obtained during elution at slow flow rate on the Sephacryl S200 column were chromatographed on a Sepharose gel filtration column using an HPLC System. The elution diagrams (Figure 6) showed that fraction 2b contained proteins with molecular weights superior to the optimal separation range of the column (Mr >300,000) and a significant proportion of peptides of low molecular weights (Mr <10,000). The fraction 3b contained a predominant peak with a molecular weight in the range from 100,000 to 70,000. The diagram corresponding to fraction 4b reveals the presence of small quantities of proteins distributed over the entire molecular weight scale. The fraction 5b (containing H3 and H4) shows a major peak with a molecular weight near 15,000.

On the SDS-PAGE patterns, the molecular weight distribution differs (Figure 7). The fraction 1b contains proteins with molecular weights in the range 50,000 to 70,000. Fraction 2b looks like a combination of 1b and 3b. The fraction 3b is characterized by two major polypeptidic components designated as P1 and P2 and having molecular weights in the range 15,000 to 17,000 for P1 and 6000 to 7000 for P2. Fraction 4b and 5b contain proteins of both high molecular weight (40,000< Mr <70,000) and low molecular weight (Mr <17,000). Fraction 5b seems to be composed of many bands in the range 70,000 to 17,000.

Figure 6. Elution profile for gel filtration fractions (2b to 5b) on the HPLC-Superose 12 HR 10/30 system in .05 M potassium phosphate, .15 M NaCl, pH 7.2 buffer (—: 2b; ---: 3b; -. -. : 4b; . . . : 5b) KDa = Kilodaltons.
particularly of a polypeptide with an electrophoretic mobility slightly inferior to P2. Only P1 was colored by the Schiff's reagent (data not shown).

Table 2 compares the amino acid composition of the fraction 3b, which is essentially composed of the Group H2, with the HFPP fraction and the total proteose-peptone. Only traces of tryptophan residue were detected. This analysis shows that HFPP and Group H2 contain cysteine and methionine, which were not detected in the total proteose-peptone fraction. Tyrosine was absent from Group H2, the proline proportion of which was half that of HFPP. Again, this was not so for the proportion of arginine.

DISCUSSION

Our recent study of the chromatographic behavior of the proteose-peptone fraction of cow's milk (19) had shown that HIC is a convenient fractionation method for proteose-peptones. The hydrophobic fraction, so isolated, containing essentially PP3, is then cleared after hydroxypatite chromatography.

The association of GPC on Sephadryl S200 with native PAGE in Tris buffer at pH 8.9 allows us to classify the HFPP components for their electrophoretic mobilities. Four groups of proteins have been listed. The molecular
TABLE 2. Amino acid composition of fraction 3b of gel filtration chromatography, hydrophobic fraction of proteose-peptone (HFPP), and whole proteose-peptone fraction (residues %).

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Fraction 3b of gel filtration</th>
<th>HFPP</th>
<th>Whole proteose-peptone</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspartic acid</td>
<td>9.0</td>
<td>9.7</td>
<td>8.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>8.7</td>
<td>7.1</td>
<td>7.0</td>
</tr>
<tr>
<td>Serine</td>
<td>10.2</td>
<td>7.5</td>
<td>8.1</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>14.6</td>
<td>14.5</td>
<td>17.8</td>
</tr>
<tr>
<td>Proline</td>
<td>4.9</td>
<td>8.6</td>
<td>10.8</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.9</td>
<td>3.7</td>
<td>3.2</td>
</tr>
<tr>
<td>Alanine</td>
<td>4.8</td>
<td>4.1</td>
<td>4.5</td>
</tr>
<tr>
<td>1/2 Cysteine</td>
<td>1.4</td>
<td>1.7</td>
<td>Trace</td>
</tr>
<tr>
<td>Valine</td>
<td>3.3</td>
<td>5.0</td>
<td>5.9</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.4</td>
<td>2.7</td>
<td>Trace</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>5.6</td>
<td>5.3</td>
<td>6.2</td>
</tr>
<tr>
<td>Leucine</td>
<td>10.3</td>
<td>11.0</td>
<td>9.3</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Trace</td>
<td>1.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.2</td>
<td>3.8</td>
<td>4.6</td>
</tr>
<tr>
<td>Lysine</td>
<td>8.6</td>
<td>7.9</td>
<td>7.8</td>
</tr>
<tr>
<td>Histidine</td>
<td>3.8</td>
<td>3.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Arginine</td>
<td>4.0</td>
<td>2.3</td>
<td>2.8</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Trace</td>
<td>Trace</td>
<td>Trace</td>
</tr>
</tbody>
</table>

Weights determination of the components of each group, by both HPLC and SDS-PAGE assays, strongly differ. Proteins of Groups H1 to H4 form aggregates that are dissociated by SDS.

Proteins of the Group H1 form aggregates with Mr >300,000 and seem essentially to be composed of high molecular weight proteins. This observation disagrees with the hypothesis to consider these components as undissociated PP3 (17). Groups H3 and H4 seem to be composed of minor components that have not been elucidated. However, the presence of proteins of high and low molecular weights are probably due to interactions of some high molecular weight molecules with Sephacryl, provoking an increase of their elution volume.

Group H2, easily isolated during GPC, attracts our attention. It contains only the electrophoretic band corresponding to PP3 defined in native electrophoresis (11, 17). It is formed of two major polypeptides of low molecular weights (17,000 and 7000), the largest of which (P1) is a glycoprotein.

Attempts to separate these proteins, involving the specific adsorption of glycoproteins on a Concanavalin A matrix, were fruitless; both proteins were eluted with the same retention time during an increasing gradient of α-methyl-D-mannoside (data not shown). This observation points out, in this context, the strong association of these polypeptides.

The antigenic identity of several PP3-enriched fractions, in particular HFPP (16) and the proteose-peptone glycoproteic fraction (11), with MFGM has been proved. The comparison of the molecular weight distribution of these last fractions with that of proteins from group H2 (Figure 8) shows that the band named P1 in this paper, seems common to both.

Figure 8. Molecular weight (MW) distribution of proteins in proteose-peptone PP3 fractions. a) Glycoprotein fraction of proteose-peptone (Kester and Brunner, 1982). b) Fraction 3b of gel filtration chromatography corresponding to the group H2.
preparations. This glycoprotein with a hydrophobic behavior, might be considered as one of the anti-MFGM reacting proteins of proteose-peptone.

ACKNOWLEDGMENT

The authors are grateful to F. Aubert for his technical assistance.

REFERENCES