Optimization for Selective Fractionation of Bovine Blood Plasma Proteins Using Poly(ethylene glycol)

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Bovine blood plasma was fractionated into fibrinogen, immunoglobulins, and albumin by poly(ethylene glycol) (PEG) precipitation. Conditions found by using centroid mapping optimization: for precipitating fibrinogen, pH 5.5, PEG 9.06%, and NaCl 1.4 M; for separating immunoglobulins (Igs) in precipitate and albumin in supernatant, PEG 12.6% and NaCl 0.35 M, which were added to the supernatant at pH 8.22, after separating fibrinogen. Immunological activity of crude IgG preparation was 90–91% of that of standard IgG and was 26% greater by precipitating at pH 8.25 than at pH 7. The separation efficiencies (separated/total protein on electrophoretogram) of fibrinogen, immunoglobulins, and albumins were 91, 88, and 92%, respectively.

In recent years there has been a growing interest in utilizing blood from sacrificed animals. This is due to the high quality of plasma proteins and the high content of nutritionally valuable iron in the blood. The prime functional properties of plasma proteins are their emulsification and binding properties (Crenwelge et al., 1974).

Weanling piglets fed blood immunoglobulins (Igs) preparations have had a faster daily weight gain, lower incidence of scours, and reduced mortality (Kennelly et al., 1979), probably due to a passive immunity (McCallum et al., 1977). Animal plasma protein is, therefore, a potential source of Igs for animal feeding as well as for human consumption.

Most popular methods for plasma protein isolation entail precipitation with ammonium sulfate or ethanol. However, these precipitation methods have a disadvantage of either requiring a high concentration or cooling to avoid denaturation. The precipitant selected for this study, poly(ethylene glycol) (PEG), is a nontoxic, water-soluble synthetic polymer, of which use as a protein precipitating agent was studied extensively by Kula et al. (1977) and Atha and Ingham (1981). Poly(ethylene glycol) has several advantages over ethanol as a fractional precipitating agent (Hao et al., 1980). In addition to being less foaming, relatively inexpensive, and nonflammable, it can be used at above zero temperature with little risk of denaturation (Polson et al., 1964). Poly(ethylene glycol) has been successfully employed for the large-scale separation of factor VIII (Johnson et al., 1971), albumin (Curling et al., 1977), and 2-macroglobulin (Wickerhauser and Hao, 1972). The fractionation of human plasma protein with PEG was reported by Polson et al. (1964) and Falksveden and Lundblad (1980).

The objective of this study was to find the best conditions for the poly(ethylene glycol) fractionation of blood plasma. Centroid mapping technique was used for best separation of blood plasma into fibrinogen, immunoglobulins, and serum albumin. Further work is under way to use the separated immunoglobulins for feeding immunologically immature young animals and the fibrinogen and serum albumin as food ingredients utilizing their characteristic functionality.

MATERIALS AND METHODS

Bovine blood was obtained from a local slaughterhouse (Intercontinental Meat Packers Ltd., Vancouver, BC). Plasma was prepared from freshly drawn blood containing 0.4% sodium citrate by centrifugation at 1020g for 15 min at 6 °C.

Plasma protein fractions derived from bovine blood (IgG, fibrinogen, 2-macroglobulin, albumin, transferrin) of high purity and poly(ethylene glycol) (3350 mol wt) were purchased from Sigma Chemical Co. (St. Louis, MO) and used as standards. Goat antibovine IgG and rabbit antibovine serum were purchased from Miles Laboratories (Elkhart, IN).

Optimization Procedure. Centroid mapping optimization (CMO) of Aishima and Nakai (1986) was used for finding the best conditions for the sequential fractionation of fibrinogen and Igs from plasma by PEG-precipitation method. The experimental conditions (factors) for the maximum separation were varied according to CMO within the following ranges: (1) for the fibrinogen, pH 4.0–9.0, PEG concentration 6–20%, and NaCl concentration 0–5.0 M; (2) for the immunoglobulins, pH 4.0–9.0, PEG concentration 10–20%, and NaCl concentration 0–2.5 M. A hand-held computer (Sharp PC-1500) with 18-kbyte RAM (Sharp Co., Osaka, Japan) was used for execution of the CMO program.

Precipitation Procedure. (*i*) *Fibrinogen Fractionation.* PEG and NaCl were added gradually to 10 mL of pH-adjusted plasma while stirring for 1 h at room temperature. The pH of plasma was adjusted with 0.1 M citric acid or 0.2 M Na_2HPO_4 solution.

(*ii*) *Immunoglobulin Fractionation.* PEG and NaCl were added gradually to 20 mL of the pH-adjusted supernatant resulted from fibrinogen precipitation (Figure 1).

Electrophoresis and Immunoelectrophoresis. Plasma protein samples were identified by the Corning agarose gel electrophoresis technique using Universal electrophoresis agarose film and a 0.05 M barbital buffer (pH 8.4).

Electrophoresis was performed at room temperature with a constant voltage of 100 V for 45 min. After electrophoresis the gel was stained in 0.1% amido black 10B for 15 min and then immersed in 5% acetic acid in methanol for 2 min. After drying at 65 °C for 15 min, the gel was transferred to a second 5% acetic acid in methanol until all excess stain was removed and then dried at 65 °C.

For evaluation of separation efficiency, SE, peak areas of Igs and other plasma proteins on the electrophoretograms were analyzed with the TG2980 scanning densitometer (Transidyne General Co., Ann Arbor, MI) at 600 nm. Separation efficiency was expressed as the ratio of the fractionated protein peak areas (IgG, fibrinogen) to total protein peak areas.

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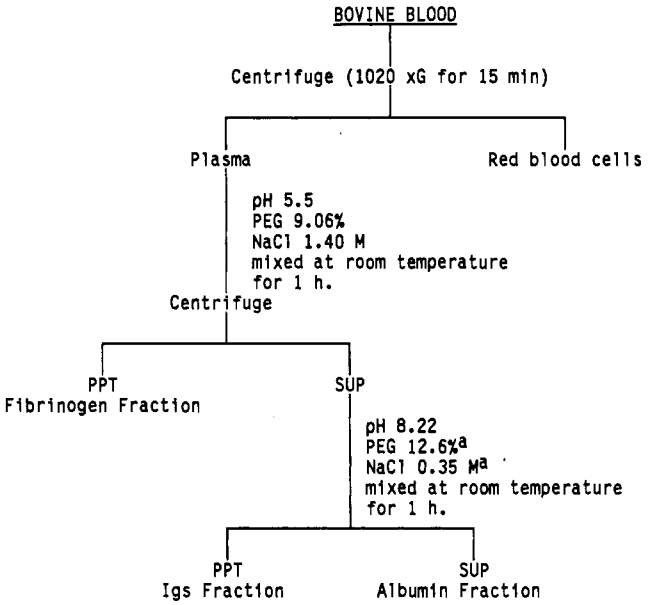

Figure 1. Flow diagram of procedures for an optimum preferential fractionation of crude fibrinogen, Igs, and albumin. PEG and NaCl concentrations shown are the amounts used in addition to the PEG and NaCl already present in the supernatant.

Immunoelectrophoresis and immunodiffusion were carried out according to the method described by Williams and Chase (1971). Immunochemical quantitative analysis of IgG was carried out by radial immunodiffusion (RID) using a kit purchased from the Miles Laboratories.

Effect of pH Difference between 7.0 and 8.25 on the Immunochemical Activity of IgG. After separation of the IgG fraction at pH 7.0 and 8.25 by adding 0.6 M phosphate buffer, the separated fraction and plasma samples similarly adjusted to pH 7.0 and 8.25 were stored at 4 °C for 5 h.

The immunochemical activity of IgG was determined by the radial immunodiffusion method. Activity of the separated IgG was expressed as a percentage against that of the standard IgG maintained at pH 7.0 and 4 °C for 5 h.

PEG Analysis. The spectrophotometric method of Childs (1975) was used.

Amino Acid Analysis. The samples were hydrolyzed with 3 N HCl at 121 °C (15 psi) for 17 h. Amino acids were analyzed by a Beckman System 6300 high-performance amino acid analyzer.

Table I. Recommended Conditions for Precipitating Fibrinogen from Bovine Blood Plasma Using Poly(ethylene glycol) by the Centroid Mapping Optimization Computer Program

vertex (exptl no.)	factors (conditions) PEG, %	NaCl, M	pH	response: SE,[a] %
1	6.000	0.000	4.000	66.120
2	19.199	1.178	5.060	74.200
3	9.299	4.714	5.060	68.540
4	9.299	1.178	8.242	17.000
5	11.499	1.964	4.707	57.200
6	19.199	0.900	4.810	65.750
7	6.754	1.329	5.196	87.030
8	16.088	2.616	5.196	67.650
9	16.088	1.329	6.354	81.850
10	12.977	1.758	5.582	84.600
11	11.940	1.472	5.711	81.870
12	10.557	1.520	5.496	87.160
13	11.824	1.583	5.597	74.090
14	8.050	2.659	5.500	77.650
15	7.500	2.200	5.800	83.520
16	6.750	1.300	5.200	63.460
17	8.500	1.300	5.400	85.170
18	9.060	1.400	5.500	90.720
19	9.560	1.800	5.600	87.000

[a] Separation efficiency (SE) = fibrinogen/total protein × 100.

RESULTS AND DISCUSSION

Optimum Condition for Precipitation. The flow diagram of a procedure selected is shown in Figure 1. PEG 4000 (approximate mol wt 3350) was used as described by Curling and Berglöf (1977).

(*i*) *Fibrinogen.* The best fractionation conditions attained after 19 experiments in centroid search were pH 5.5, PEG 9.06%, and NaCl 1.40 M (Table I; Figures 1 and 2), resulting in a precipitate with a separation efficiency of 91%. The resulting mapping, i.e.. response surface pattern (Figure 2), of each factor clearly indicates vertex 18 is the optimum point.

(*ii*) *Igs.* The experiments were carried out by adjusting pH (4.0–9.0), PEG (10–20%), and NaCl (0–2.5 M). After 21 experiments in centroid search, the best fractionation conditions were found to be pH 8.22, PEG 12.6%, and NaCl 0.35 M (Table II; Figures 1 and 3), resulting in a separation efficiency of 88% Igs in the precipitate and of 92% albumin in the supernatant. The mapping of each

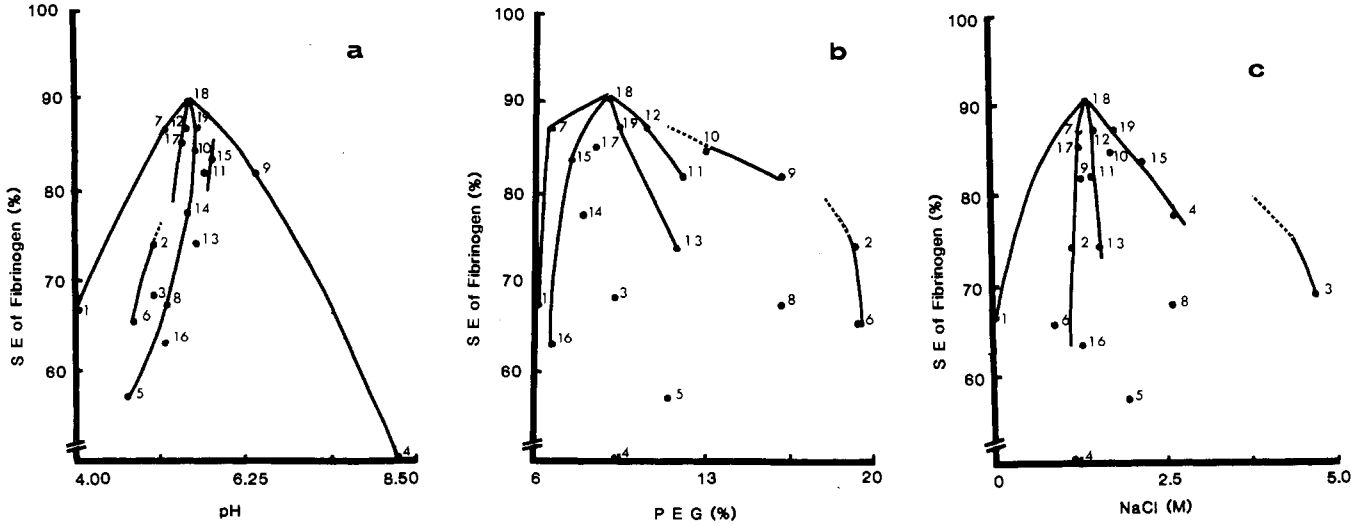

Figure 2. Response surface patterns for pH (a) and PEG (b) and NaCl (c) concentrations obtained by mapping data from the centroid optimization for fibrinogen. Numbers beside each point correspond to the vertex number in the CMO.

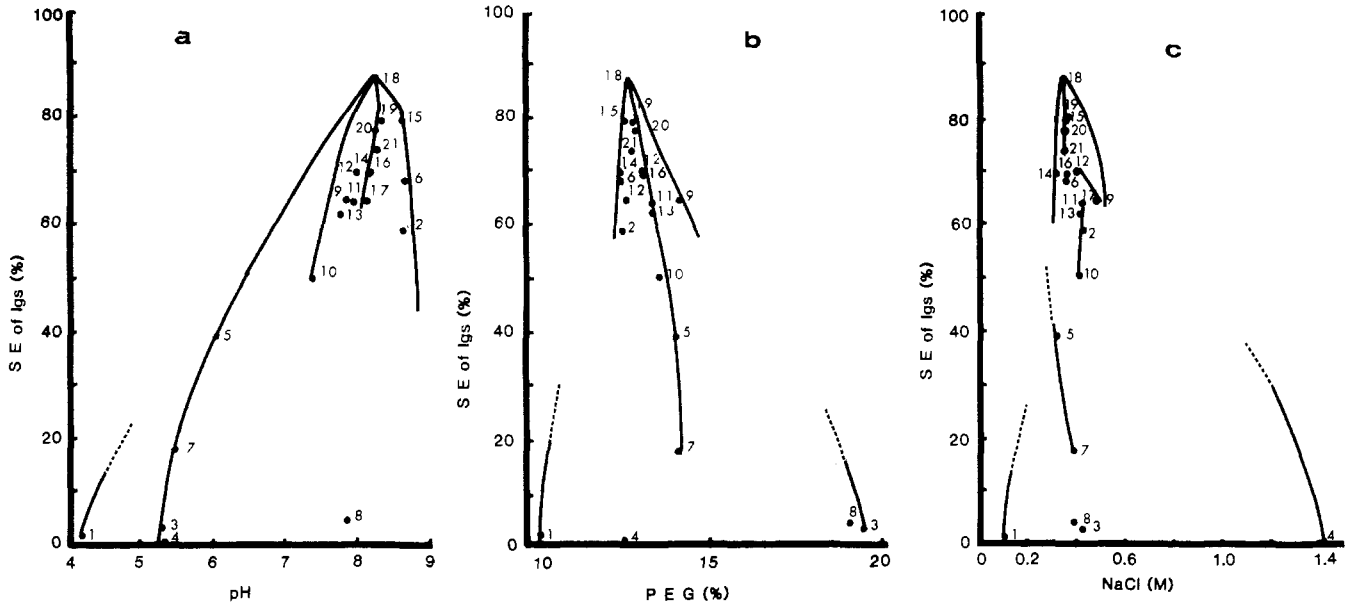

Figure 3. Response surface patterns for pH (a) and PEG (b) and NaCl (c) concentrations obtained by mapping data from the centroid optimization for Igs. Numbers beside each point correspond to the vertex number in the CMO.

Table II. Recommended Conditions for Separating Igs and BSA from Bovine Blood Plasma Using Poly(ethylene glycol) by the Centroid Mapping Optimization Computer Program

vertex (exptl no.)	factors (conditions)			response:
	PEG, %	NaCl, M	pH	SE,[a] %
1	10.000	0.100	4.200	2.421
2	12.357	0.429	8.631	58.900
3	19.428	0.429	5.307	3.860
4	12.357	1.419	5.307	1.120
5	13.928	0.319	6.046	39.500
6	12.357	0.359	8.631	68.040
7	14.023	0.392	5.497	18.440
8	19.023	0.392	7.847	5.120
9	14.023	0.491	7.847	64.600
10	13.468	0.414	7.352	50.360
11	13.282	0.422	7.934	64.120
12	13.036	0.399	7.963	69.690
13	13.262	0.412	7.741	62.120
14	12.357	0.319	7.963	69.570
15	12.517	0.359	8.592	79.360
16	12.997	0.359	8.120	69.050
17	12.517	0.481	8.120	64.510
18	12.623	0.346	8.225	88.010
19	12.712	0.355	8.312	79.140
20	12.777	0.353	8.219	77.490
21	12.704	0.351	8.252	73.630

[a] Separation efficiency (SE) = immunoglobulins/total protein × 100.

factor (Figure 3) clearly indicates that vertex 18 is the optimum point for Igs separation.

On the basis of studies with purified proteins, it was reported that the dependence of solubility of proteins on PEG concentration was exponential (Middaugh et al., 1979; Atha and Ingham, 1981). As shown in

$$\log S = \log S_0 - K[\mathrm{PEG}]$$

semilog plots of solubility data are usually linear, analogous to the salting-out phenomenon (Cohn and Ferry, 1943), where S and S_0 are the solubilities in the presence and absence of PEG, respectively. With PEG, the slope K is a constant influenced by the amount of the protein and/or the amount of the PEG. Thus, manipulation of parameters such as pH, temperature, and ionic strength will improve the separation of two proteins to the extent that their intrinsic solubilities appreciably differ. Poly(ethylene glycol) is an inert molecular sponge, of which the interior is permeable to solvents but not to proteins and which indiscriminately raises the effective concentrations of proteins. Polson et al. (1964) found that in PEG separations of γ-globulin and fibrinogen from human plasma, pH was the most critical factor, ionic strength had little effect, and results were best when used at around 20 °C. However, according to Chun et al. (1967) ionic strength affected the precipitation of human serum albumin and γ-globulins.

Complete removal of PEG from the PEG-precipitated proteins is important in the utilization of the separated proteins. The molecular weight of PEG, 3350, is much lower than that of most proteins of interest, but its random-coil structure gives it a relatively large exclusion radius hindering its removal by dialysis, ultrafiltration, or gel filtration (Hao et al., 1980; Hellsing, 1968). However, Busby and Ingham (1980) reported a successful removal of PEG 4000 with a microporus ultrafiltration membrane, which was not affected by the presence of albumin between 1 and 100 mg/mL. PEG can be also removed from protein solutions by using DEAE- or CMC-cellulose at pH 6.9, as it is not retained by the column (Ryle, 1965). Since the hydrophilic polymer is free of charge, it has no affinity to the column. This approach is easily integrated into the purification scheme. Another approach to this problem involves the use of salts to induce the separation of PEG solutions into two aqueous phases (Hao et al., 1980). This cause justifies the inclusion of NaCl in the optimization of preferential fractionation of plasma proteins. For further purification, the crude IgG fraction of plasma proteins was subjected to affinity chromatography using tris(carboxymethyl)ethylendiamine–Sepharose 4B (TED–Sepharose 4B) loaded with Ni and Cu tandem columns (Lee and Sim, 1985; Porath and Olin, 1983). The PEG content of PEG fraction was 0.01% after the column chromatography. Poly(ethylene glycol)-precipitated Igs from porcine colostrum are presently used in feeding trials with piglets (Drew, 1986).

Electrophoresis and Immunoelectrophoresis. Agarose gel electrophoresis was used for the rapid quantification of plasma protein, and relatively sharp peaks were shown for Igs, albumin, and fibrinogen (Figure 4).

For the evaluation of the immunological function of plasma protein, immunoelectrophoretic analysis and radial

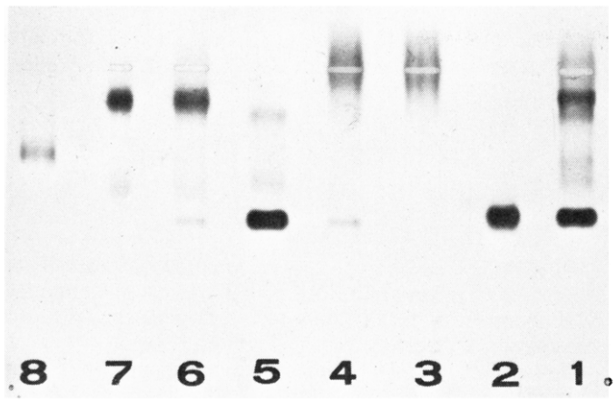

Figure 4. Comparison of bovine plasma protein component by agarose gel electrophoresis: (1) plasma; (2) bovine serum albumin (BSA); (3) immunoglobulin G (IgG); (4) immunoglobulins fraction (IgS); (5) BSA fraction (supernatant); (6) fibrinogen fraction; (7) fibrinogen; (8) α_2-macroglobulin.

Table III. Effect of pH on the Immunochemical Activity of Crude IgG Fraction and Plasma

sample	immunochemical act. of IgG, %	
	pH 7.0	pH 8.25
standard IgG	100	126
crude IgG fraction	90.8	113.4
plasma	47.8	41.0

immunodiffusion were used and the results are shown in Figures 5 and 6.

Immunochemical activity of IgG was not detected in the BSA fraction; however, there were some precipitant lines in both the fibrinogen fraction and the standard fibrinogen (see Figure 6). It is very difficult to isolate fibrinogen completely from other plasma proteins without contamination with IgG.

Effect of pH Difference on the Immunochemical Activity of IgG. The immunochemical activity of crude Igs preparation was compared at pH 7.0 and 8.25, and the results showed that the immunochemical activity of IgG at pH 8.25 was 26% higher than at pH 7.0 by RID (Table III). Immunological activity of crude IgG preparation was 90 and 91% of that of the standard IgG at pH 8.25 and pH 7.0, respectively. It was concluded that pH 8.25 was preferable for the precipitation method for the selective separation of Igs from plasma proteins while maintaining

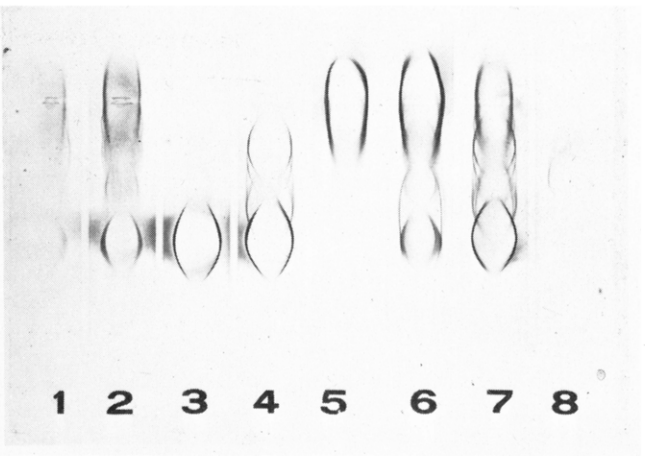

Figure 5. Immunoelectrophoresis of plasma preparations: (1) fibrinogen; (2) crude fibrinogen preparation; (3) BSA; (4) supernatant obtained at the optimum separation condition (BSA fraction); (5) IgG; (6) precipitate (crude Igs fraction); (7) bovine plasma; (8) α_2-macroglobulin.

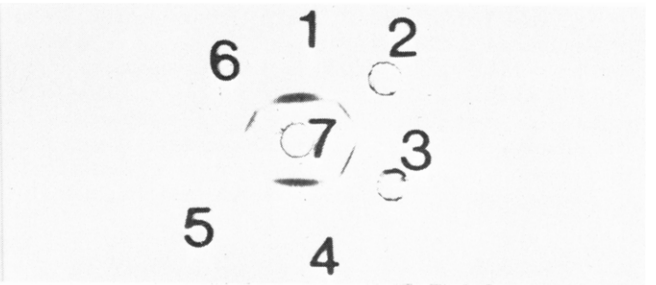

Figure 6. Immunodiffusion of plasma preparations: (1) IgG; (2) bovine plasma; (3) fibrinogen; (4) crude Igs fraction (precipitate) obtained at the optimum conditions; (5) BSA fraction (supernatant); (6) crude fibrinogen fraction.

the activity of IgG during the fractionation. These results indicate a higher pH for the optimum fractionation of IgG than that reported by Hao et al. (1980), Polson et al. (1964), and Falksveden and Lundblad (1980).

Amino Acid Analysis. The amino acid compositions of the plasma, the crude fibrinogen fraction, Igs fraction, albumin fraction, and standard proteins (IgG, BSA, fibrinogen) are compared in Table IV. Each fraction has a similar pattern as the standard protein. The most abundant amino acids from plasma and plasma fractions

Table IV. Amino Acid Composition (% Total Amino Acid) of Bovine Plasma (BP), Crude Fibrinogen Fraction (CFF), Crude Immunoglobulins Fraction (CIF), Crude Albumin Fraction (CAF), Bovine IgG, Albumin (BSA), and Fibrinogen (FIB)

amino acid	BP	CFF	CIF	CAF	IgG	BSA	FIB	IBP[a]
Asp	10.0	9.5	10.1	9.8	10.5	9.0	10.8	10.1
Thr	6.4	7.0	5.6	5.4	7.6	5.0	5.8	8.5
Ser	6.4	7.7	4.8	4.6	8.0	3.6	5.1	9.5
Glu	13.4	12.6	14.4	13.8	12.1	14.9	14.1	14.1
Pro	5.3	5.7	4.8	4.7	6.1	5.2	5.2	6.4
Gly	3.8	4.2	2.6	2.6	5.2	1.8	3.8	3.3
Ala	4.6	4.4	5.2	5.2	3.6	5.4	4.4	5.1
Cys	2.6	1.7	3.2	3.4	1.8	4.0	2.8	2.1
Val	6.0	7.2	5.7	5.4	6.5	5.2	4.8	5.3
Met	1.2	1.3	0.4	0.9	1.6	0.9	1.5	1.1
Ile	2.8	3.0	2.5	2.6	3.2	2.2	3.0	2.9
Leu	9.0	8.7	10.4	10.6	7.0	10.6	8.6	9.2
Tyr	5.0	5.4	4.8	5.3	5.4	5.0	5.0	4.7
Phe	5.1	4.4	6.2	6.5	4.2	6.0	4.9	4.5
His	3.6	3.4	4.2	4.2	2.8	4.2	3.6	3.0
Lys	9.1	8.2	10.2	10.3	7.8	11.7	10.2	9.4
Arg	5.8	5.6	5.0	5.3	6.6	5.4	6.6	5.4
NH_3	0.8	1.1	1.4	0.8	1.8	0.9	1.5	

[a] IBP = isolated beef plasma (spary dried) by Pals (1970).

are glutamic acid, aspartic acid, lysine, leucine, and valine.

The selective fractionation method reported in this paper is simple and thus appears to be suitable for a large-scale plasma fractionation.

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Effect of Methods To Remove Polyphenols from Sunflower Meal on the Physicochemical Properties of the Proteins

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Sunflower meal was extracted four times with 40% aqueous acetone, 40% aqueous ethanol, or 40% aqueous methanol to remove polyphenols. The flaked kernels were also extracted five times with water (pH 6.0) at 70 °C. With organic solvents the efficiency of polyphenol removal was 95–98% whereas with water it was only 83%. Extraction with water and organic solvents increased the protein content of the meal and decreased the total sugar content. The amino acid composition of the protein did not change. Nitrogen solubility of the extracted meals was lower than that of the unextracted meal in water in the range pH 2–8. However, there was no change in the gel filtration or sedimentation velocity pattern. In polyacrylamide gel electrophoresis, methanol-extracted meal proteins had higher mobility. Between 300 and 400 nm the fluorescence emission intensity of extracted meal proteins was higher than that of the unextracted meal proteins. Extracted meal proteins were hydrolyzed to a greater extent by trypsin/α-chymotrypsin/papain than the unextracted meal proteins.

Sunflower (*Helianthus annuus*) is an important source of vegetable oil, and the meal after extraction of oil is a valuable source of proteins (Sosulski and Bakal, 1969). Sunflower proteins have unique organoleptic and functional properties that would make them useful in processed foods (Sosulski, 1979). There are no known toxic constituents and antinutritional factors in sunflower meal (Clandinin, 1958). The major difficulty in the utilization of sunflower meal in human diets is the presence of hulls and polyphenols in the seed. The hulls contribute to high crude fiber content of the meal. The presence of polyphenols causes the meal and the proteins to become colored during alkaline extraction of the proteins (Smith and Johnson, 1948): the polyphenols are colorless at low and neutral pH values and assume greenish yellow to brown color in the range pH 8.0–11.0. They also lower the nutritive value (Jung and Fahey, 1981), since they interact with essential amino acids such as lysine and methionine

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