Isoelectric Focusing of Bovine Colostrum Immunoglobulins

R. V. JOSEPHSON, E. M. MIKOLAJCIK, and V. K. SINGH
Department of Food Science and Nutrition
The Ohio Agricultural Research and Development Center and
The Ohio State University, Columbus 43210

Abstract

Isoelectric focusing in acrylamide gels and sucrose gradient columns was used to study the heterogeneity of bovine colostrum immunoglobulins. The immunoglobulins were focused into a number of individual protein species with isoelectric points of from pH 4.6 to 6.7. Protein species exhibiting different isoelectric points also exhibited heterogeneity by immunoelectrophoresis. Re-focusing of the individual column fractions indicated that each major isoelectric protein was a homogeneous species and not an artifact. Individual classes and subclasses of the immunoglobulins were not identified.

Introduction

Several recent communications have dealt with the heterogeneity of immunoglobulin classes and subclasses isolated from human and animal species (3, 4, 10, 17, 29). Classification of immunoglobulins into classes and subclasses has been based on their antigenic and physical-chemical characteristics (3, 4, 10, 29). Bovine serum and lacteal secretions have three major antigenically distinct classes of immunoglobulins IgM, IgG, and IgA (3, 4, 29). The IgG class contained at least two subclasses, IgG1 and IgG2, and likely others (21, 29). Moreover, there are recent reports (2, 3) of genetic variation within IgG.

Purification and characterization of bovine lacteal immunoglobulins in our laboratory (22, 30) have utilized a crude immunoglobulin preparation, Fraction D, obtained by Smith's ammonium sulfate fractionation (31). Characterization of this fraction by disc gel electrophoresis, immunoelectrophoresis, and analytical ultracentrifugation has revealed IgG and its subclasses, IgG1 and IgG2, IgM, and occasionally IgA (22, 30) whereas blood serum albumin and other milk serum proteins were not detected.

In light of the current need to study the physical, chemical, and biological properties of the bovine lacteal immunoglobulins, it is important to ascertain by the best available means their purity and heterogeneity (3, 17, 28). One approach for study of the heterogeneity of γ-globulins from human (5, 11, 18, 24, 32), mouse (26), and rabbit (6, 7, 8, 13) species has been by isoelectric focusing on sucrose gradient columns or on acrylamide gels. The procedure was particularly effective with immunodiffusion or immunoelectrophoresis (5, 6, 7, 9).

Based upon these reports, isoelectric focusing in combination with immunoelectrophoresis was used to study Fraction D immunoglobulins from bovine colostrum whey.

Experimental Procedure

Fraction D was prepared from bovine colostrum whey by the method of Smith (31), dialyzed against water to remove nonprotein substances, and lyophilized. Antisera used for immunoelectrophoresis were rabbit anti-bovine serum from Dexter Biologicals, Dexter, Michigan and rabbit anti-bovine colostrum Fraction D prepared in our laboratory (30).

Glass-distilled deionized water was used in all procedures.

Column isoelectric focusing. Experiments were performed at < 10°C in a special glass column (LKB 8101) of 110 ml capacity according to instructions in the LKB manual (25) with some modifications (12, 19). A linear sucrose gradient solution of 0 to 47% (w/v), formed by a gradient mixer (LKB 8121) contained 1% carrier ampholytes (LKB, pH 5 to 8) and 20 mg of bovine colostrum Fraction D. The light and dense electrode solutions were 1% NaOH (cathode) and 1% H₂SO₄ (anode). Ribonuclease-free crystalline sucrose (Nutritional Biochemicals Corp.,

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Cleveland, Ohio) was used to form the density gradients (14).

Focusing was established and maintained at <2.0 w (25) with an electrode potential of 500 v for 48 hr, then the gradient was emptied by gravity flow and collected in 2-ml fractions.

The pH of column fractions was determined using a Corning Model 12 Expandomatic pH Meter with a combination glass electrode having a relative accuracy of .01 pH units. pH was measured at 24 ± 1 C as suggested earlier (19). Light absorption at 280 nm estimated protein in column fractions even though some fractions were slightly turbid.

Focused fractions were dialyzed against water for 72 hr to remove nonprotein substances and then lyophilized. Dry weights of the lyophilized fractions which also served as a measure of protein were made with a Mettler single-pan analytical balance having a sensitivity of .1 mg. Lyophilized fractions were dispersed in water. Portions were subjected to gel isoelectric focusing and immunoelectrophoresis.

**Gel isoelectric focusing.** The method of Wrigley (33) with reported modifications (11, 13, 15, 27) was used for resolution of the bovine immunoglobulins. The stock solutions were: N, N, N', N'-tetramethylethylene diamine (.8% v/v in water), .75 ml; riboflavin (.004% w/v in water), 1.00 ml; acrylamide (21.75% w/v in water), 2.00 ml; sucrose (40% w/v in water), 2.72 ml; carrier ampholytes (40%, LKB, pH 5-8), .22 ml; and bovine colostrum Fraction D in water, 2.01 ml.

The final gel preparation of 8.70 ml contained 5% total gel at 5% cross-linkage (13), 12.5% sucrose, 1% carrier ampholyte, and 770 to 970 µg Fraction D.

Approximately 1.35 ml of a well mixed, evacuated gel solution was added under subdued light to a series of vertically held .5 x 7.5 cm glass tubes. They were sealed at the bottom by Saran Wrap and fitted into punctured rubber adapters to avoid suction forces. The tubes were then overlaid with water and photo-polymerized by a fluorescent light for 1 hr at <10 C. Following removal of the Saran Wrap, tubes were mounted in numbered grommets of the top vessel of the disc electrophoresis apparatus (Canalco, Rockville, Maryland). The top (cathodic) and bottom (anodic) vessels were filled with aqueous solutions of cold 0.4% ethanolamine and .02% H₂SO₄. Focusing was established at .5 mamp/tube (6 mamp/12 tubes) and voltage increased to the desired potential of 200 v as the current decreased during the period of 1 hr. The run was continued for 8 hr at 10 C.

The electrolyte concentrations approximated a pH gradient of 5 to 8 with the LKB carrier ampholytes. Attempts to use lower concentrations of electrolytes approaching ampholyte pH limits as suggested by Percival et al. (27) permitted optimum pH gradient formation but did not resolve the immunoglobulins of Fraction D. Since an initial voltage of 200 to 300 v could be used without exceeding a current of .5 mamp per tube, it was possible that there was insufficient electrical conductivity for protein resolution within the 8 hr run.

Gels 7.3 cm long were removed in ice water bath by rinsing the tubes with a hypodermic needle. Selected gels were stained by the method of Awdeh (1) in a bromophenol blue solution for 1 to 2 hr, destained until the background was clear, usually 1 to 2 days, and then photographed.

The unstained gels were cut with a razor blade into fourteen .5 cm slices and a final slice of .3 cm. The slices were crushed with a glass rod and dispersed in .5 ml of water and pH determinations made as described for the column fractions. The .5 cm slices were used because .16 cm slices prepared with a Canalco lateral gel slicer yielded an erratic pH gradient. It was believed that the ampholyte (1%) in each .15 cm slice was not sufficient to buffer the water diluent to yield a representative pH. Subsequently, increasing the ampholyte to 2% did not significantly overcome the problem.

**Immunoelectrophoresis.** By modification of the procedure of Catsimpoolas (7, 9), the focused gel slices were subjected to immunoelectrophoresis. Focused gels removed from the tubes were sliced into 40 to 45 slices with a Canalco lateral gel slicer. Each slice was placed into a well of a spot test plate, crushed with a glass rod, wetted with ca. 5 µliters of .05 m barbitone acetate buffer, pH 8.6, and transferred into the agar slide sample well (6 mm diameter) which had been prepared with a cork borer under vacuum so as to accommodate the entire gel slice. Approximately 15 µliters of the barbitone acetate buffer were added to each sample well containing the gel slice.

For immunoelectrophoresis of the isoelectric focusing column fractions, the dialyzed, lyophilized samples were dispersed in 1.0 ml of water and a 5-µliter aliquot was placed into the sample well (2 mm diameter) of the agar slide.

Agar gel slides containing Oxoid ID agar and .025 M Oxoid barbitone acetate buffer were
used for all immunoelectrophoresis runs as described (23). Slide trays for immunoelectrophoresis were placed in a Colab Universal electrophoresis chamber connected to a Colab Vulcan Mark III power supply and run at 18 mamp and 120 v for 90 min with .05 m barbitone acetate buffer, pH 8.6. Procedures for addition of antisera, washing with saline, staining and destaining and photography have been described (23).

Results and Discussion

Crude bovine immunoglobulins prepared from colostrum whey by ammonium sulfate fractionation (Fraction D) were isoelectric-focused in acrylamide gels and sucrose gradient columns. Data are presented for each isoelectric focusing system and the immunogenic properties of the fractions determined by immunoelectrophoresis.

Gel isoelectric focusing. Preliminary experiments determined the resolving ability of LKB carrier ampholytes at pH 5 to 8, 3 to 6, and 7 to 10. For Fraction D, carrier ampholytes at pH 5 to 8 were selected because they produced superior resolution.

Figure 1 shows a typical isoelectric focused gel (IEF-gel) pattern of Fraction D with pH's of the .5-cm slices from the gel. A number of discrete and diffusely stained bands were noted in the approximate pH range of 4.9 to 6.7.

The immunoelectrophoretic (IME) patterns of Slices 7 through 35 from an unstained IEF-gel are in Figure 2. The patterns were developed with anti-bovine serum. Immunoelectrophoretic patterns developed with anti-bovine colostrum Fraction D were similar and are not shown.

Although somewhat faint in some instances, precipitin arcs were distinguishable in Slices 9 through 34. Faintness of the arcs was probably related to a low concentration of protein in some of the slices. However, differences were observed in precipitin arcs in relation to the IME sample well. Starting with Slice 9, the arc location progressed steadily from anode to cathode indicating that the immunoglobulin species resolved by gel isoelectric focusing were also distinguishable by immunoelectrophoresis.

Column isoelectric focusing. In addition to experiments with IEF-gels, bovine colostrum Fraction D was also focused by sucrose gradient column isoelectric focusing (IEF-column) which enabled focusing preparative amounts (20 mg) of Fraction D and direct pH measurement of eluted fractions.

Absorbance at 280 nm (Curve a) and pH profiles (Curve b) of focused column fractions are in Figure 3. For absorbance at 280 nm, the patterns reveal two major peaks (Fractions 10 to 17 and 18 to 22), a shoulder extending from the second peak (Fractions 23 to 26), and two minor peaks (Fractions 27 to 30 and 31 to 37). The pH gradient compares favorably with that formed in the IEF-gel in Figure 1 covering pH 4.5 to 7.7.

Analysis of each column fraction by gel isoelectric focusing (Fig. 4) revealed that most of Fractions 11 to 36 of pH from 4.6 to 6.7 contained isoelectrically distinct protein bands. Relative band location in Fractions 11 to 36 appears to correspond to that of components resolved by the IEF-gel of Fraction D (a, Fig. 4). However, the pH (pI) values for the protein band locations (Fig. 1) were about .3 pH unit higher than those determined for the original column fractions (Fig. 3). The discrepan-

Fig. 1. pH gradient and gel pattern obtained upon isoelectric focusing (IEF) of bovine colostrum Fraction D.
Fig. 2. Immunoelectrophoretic (IME) patterns of .16-cm gel slices of an isoelectric focusing (IEF) gel of bovine colostrum Fraction D developed with rabbit anti-bovine serum. Slices 7 through 35 are on the right and the IME pattern for Fraction D is labeled a. Arrows indicate location of the slice in the intact stained gel on the right.
Fig. 3. Absorbance a and pH b profiles of 2-ml fractions collected after isoelectric focusing of bovine colostrum Fraction D for 48 hr on a sucrose gradient column.

Immunoelectrophoretic (IME) patterns of Fraction D (Sample a) and IEF-column fractions of Fraction D developed with anti-bovine serum are in Figure 4. As was observed the precipitin arc for each fraction appears to differ in location relative to the IME sample well with migration towards the negative pole. While not presented, similar immunoelectrophoretic patterns were obtained with anti-bovine colostrum Fraction D. Fractions 35 and 36 showed faint IEF-gel bands but yielded no immunological activity against anti-bovine serum or anti-bovine colostrum Fraction D. It was not determined whether lack of immunological response by these and other proteins in earlier fractions was related to protein concentration, potency of antiserum, or specificity of antisera.

Until further work is performed with known standards, monospecific antisera, and other criteria, it seems inappropriate to speculate on the class or subclass of immunoglobulins represented by the various precipitin arcs.

Visible rings of precipitation were formed during isoelectric focusing on the sucrose gradient column. However, during gravity collection, the precipitin zones appeared to disperse into solution, although some fractions (11 to 16) remained slightly turbid. Incorporation of ethylene glycol (18) or nonionic detergents into the sucrose gradient may be necessary to minimize precipitation of bovine colostrum Fraction D immunoglobulins.

One concern has been the possible formation of artifacts during isoelectric focusing. Certain acidic proteins such as wool proteins (16), bovine plasma albumin, and mercaptalbumin (20) complex with ampholyte components. Kaplan and Foster (20) found that bovine plasma albumin had a major component at pH 5.28 and a minor component at pH 4.7 to 4.8 by column and gel isoelectric focusing. However, the minor component was an artifact when it reappeared upon subsequent re-focus-
FIG. 4. Isoelectric focusing (IEF) gel and immunoelectrophoretic (IME) patterns of Fractions 9 through 36 collected after isoelectric focusing of bovine colostrum Fraction D for 48 hr on a sucrose gradient column. Fraction D is labeled a.
ing of the major component.

Consideration was given to the possibility of artifact formation with the bovine immunoglobulins. Re-focusing of the IEF-column fractions in acrylamide gels resulted in discrete homogenous protein bands with pH values similar to those of the original fractions. That there were no major bands generated with common pH values suggested that the major protein bands of bovine colostrum immunoglobulins are discrete protein species and are not complexes of protein with ampholyte. Leaback and Rutter (24) reached a similar conclusion by re-focusing human γ-globulins. However, the few weak bands on IEF-gels in addition to the major discrete band, especially in Fractions 24 to 36, could have resulted from an artifact.

In summary, isoelectric focusing of bovine colostrum immunoglobulins in acrylamide gels or sucrose gradient columns has revealed their heterogeneity in isoelectric (pI) points and immunoelectrophoretic reaction. Similar findings were observed by Catsimpoolas (6, 8) for rabbit γ-globulins and Murgita and Vas (26) for mouse γ-globulins. However, the bovine immunoglobulin preparation covered only approximately 2.0 pH units of from 4.6 to 6.7 and was lower than those reported for other mammalian immunoglobulins. For example, human serum γ-globulins (18) were of from pH 6.7 to 8.5, whereas rabbit γ-globulins (7) were from pH 6 to 8, and mouse γ-globulins (26) from pH 6.3 to 8.0. Whether differences in pI values between bovine and human γ-globulins are related to type and concentration of the immunoglobulin class is not known. The principal immunoglobulin of human milk is IgA, whereas for bovine milk it is IgG (17).

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