Isolation and characterization of factor I of the bovine complement system

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Objective—To isolate and characterize factor I of the bovine complement system.

Sample Population—Serum obtained from the blood of beef cattle.

Procedures—Serum samples were fractionated to yield factor I by means of sequential precipitation, ion-exchange, and gel-filtration chromatography. The protein was identified throughout the procedure on the basis of its ability to degrade the α′-chain of bovine C3b in the presence of bovine factor H. Electrophoresis in polyacrylamide gels was used to assess the degradation of C3b and determine the molecular weights of factor I and its component polypeptide chains.

Results—Bovine factor I had an apparent molecular weight of 94 kd and consisted of 2 disulfide-bonded polypeptides that had apparent molecular weights of 51 and 42 kd (under reducing conditions). Factor H was required for the factor I cleavage of the α′-chain of bovine C3b into iC3b. A similar cofactor effect was provided by trypsinized bovine erythrocytes or erythrocyte ghosts. Bovine properdin was prepared and shown to be a single polypeptide chain of 58 kd in the reduced form.

Conclusions and Clinical Relevance—Bovine factor I can be purified from serum by a simple 4-step procedure. It is structurally and functionally comparable to factor I of other species, and its purification completes the isolation and characterization of all the soluble components of the bovine alternative complement pathway. (*Am J Vet Res* 2003;64:989–993)

Cattle share many immunologic features and disease syndromes with other mammals, yet the ruminant immune system has some unique features, particularly regarding the humoral aspect of the immune response.[1] This may prove to be of importance in studies of the relationship between the complement system and disease. Evidence for the role of the **alternative complement pathway (ACP)** activation in the immune response of cattle to various pathogens has been noted.[2-5] Further purification and characterization of bovine complement components may aid in defining the association between the ACP and inflammatory and defensive reactions of bovine immune response mechanisms.

Factor I is an endopeptidase composed of 2 covalently linked polypeptides: a serine protease light chain and a heavy chain.[6] The enzyme circulates in plasma in an activated, rather than proenzymatic, form. Factor I has a very limited substrate range that encompasses C3b, C4b, C3i, and C4i. The role of factor I is to regulate the activities of the C3 and C5 convertase enzymes by the proteolytic cleavage of the C3b and C4b subunits in the presence of appropriate cofactors. Inactivation of human C3b to iC3b requires the soluble factor H, C4b binding protein, membrane-associated CR1 (CD35), or the **membrane cofactor protein (MCP** [CD46]); the process involves cleavage of the C3b α′-chain to form 2 fragments (molecular weights, 68 kd and 43 kd) that are covalently linked to the β-chain. The purpose of the study reported here was to isolate and characterize bovine factor I.

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Materials and Methods

Samples—Blood was obtained from beef cattle at a local abattoir. Erythrocytes were obtained from blood that was collected into an anticoagulant. Bovine serum was separated from coagulated blood samples. Human serum was obtained from blood samples donated by individuals in our laboratory. Bovine C3 and complement factors B, D, and H were isolated, as described.[7-10] Human factor I was acquired as a commercial preparation.[a]

Solutions—**Gelatin-barbital-buffered saline (GBS)** solution consisted of barbital-buffered saline solution (1.02 g of sodium diethylbarbiturate/L and 8.3 g of NaCl/L) at physiologic ionic strength (pH, 7.2) containing 0.1% gelatin. A GBS solution containing 15mM **ethylene glycol bis-amino tetraacetate (EGTA)** and 4mM $MgCl_2$ was designated GBS-EGTA. Physiologic saline (0.9% NaCl) solution buffered with phosphate (pH, 7.2) was designated PBS solution.[7]

Isolation of bovine complement factor I—Bovine factor I was prepared by a 4-step procedure on the basis of methods used for the purification of human factor I.[11,12] Briefly, 1 L of bovine serum was dialyzed for 16 hours at 4°C against 0.02M sodium phosphate (pH, 8.0) containing 0.01M EDTA and 0.12M NaCl and adsorbed with 600 mL of diethyl[2-hydroxypropyl]aminoethyl-complex,[b] which had been equilibrated with the aforementioned buffer. This procedure removed proteins that had a negative charge at this pH. The unadsorbed protein fraction was precipitated with $(NH_4)_2SO_4$. The precipitate formed between 40 and 60% saturation of $(NH_4)_2SO_4$, was dissolved in and then dialysed against 0.02M potassium phosphate (pH, 6.0) containing 0.02M NaCl, and chromatographed on a column of carboxymethyl-complex[c] equilibrated with this buffer. Factor I was eluted by a linear gradient of 0.02 to 0.20M NaCl, followed by 1M NaCl. The fractions containing factor I, determined by cofactor-mediated cleavage of bovine $C3b(H_2O)$,[10] were pooled and concentrated by precipitation with $(NH_4)_2SO_4$. The precipitate was harvested by centrifugation at 4°C and 8,000 × *g* for 20 minutes and dissolved in PBS solution. It was applied to a column of cross-linked copolymer of allyl dextran and N,N′-methylene-

bisacrylamide[d] (2.5 × 90 cm) equilibrated in the same buffer. The fractionation range of the column was 5 to 250 kd, and the elution buffer was PBS solution. The factor I eluted as a single symmetric peak and was harvested and stored at –70°C.

Isolation of bovine complement C3b as C3b(H_2O)—Cobra venom (CVF), isolated from *Naja naja kaouthia*,[e] was coupled to a cyanogen bromide-activated matrix of cross-linked beaded agarose[f] at a ratio of 5 mg CVF:1 mL matrix volume.[7] Aliquots (100 µL) of a 50% suspension of the agarose matrix-CVF in PBS solution containing 15mM EGTA and 4mM $MgCl_2$ were converted to agarose matrix-CVF,Bb by incubation with 7.8 µg of factor B and 0.1 µg of factor D at 37°C for 30 minutes. The agarose matrix-CVF,Bb was pelleted by centrifugation at 3,000 × *g* for 10 minutes and washed at 22°C by repeated suspension in 20 volumes of PBS solution. The washed, packed agarose matrix-CVF,Bb samples were immediately added to 30 µL of C3 (10.5 µg) containing 10mM magnesium acetate, and the mixtures were incubated at 37°C for 45 minutes. Aliquots of 25 µL were removed and pooled after centrifugation at 3,000 × *g* for 10 minutes. The C3b(H_2O) was separated from C3a by passage through a column of cross-linked copolymer of allyl dextran and N,N′-methylene-bisacrylamide.[d]

Sodium dodecyl sulfate PAGE was performed in slab non gradient or linear gradient gels by means of a discontinuous Tris-glycine buffer system.[13] Coomassie brilliant blue[g] stain was used to stain gels; estimates of molecular weight were obtained by comparison with markers of known molecular weight.[g]

Isolation of properdin—A modification of methods for the isolation human properdin was used to prepare bovine properdin.[14] Briefly, 1,600 mL of bovine serum was mixed with 150 g of a yeast cell wall preparation for 45 minutes at 37°C.[15] The yeast-cell-wall preparation was harvested, washed once with PBS solution, and eluted with PBS solution containing 1M NaCl for 5 hours at 22°C and then for 16 hours at 4°C. After centrifugation at 3,000 × *g* and 4°C for 30 minutes, the supernatant was dialyzed against 0.02M Tris-HCl (pH, 8.4) containing 0.03M NaCl and passed through a 5- × 15-cm column of cross-linked, beaded agarose[h] equilibrated with the Tris buffer. Properdin passed through the column, and the breakthrough fractions were collected, pooled, and concentrated by ultrafiltration. The major contaminant, bovine IgG, was removed by passing the properdin preparation through a column of protein G from group C *Streptococcus*[i] and subsequently through a column of allyl dextran and N,N′-methylene-bisacrylamide[d] equilibrated with PBS solution. Activated properdin was detected by standard procedures.[15]

Bovine erythrocyte preparations—Blood from cattle was collected into anticoagulant solution containing heparin (2.5 units/mL) in 2.85% trisodium citrate with 0.01M benzamidine[e] and 0.01% soybean trypsin inhibitor.[e] For the preparation of erythrocyte membranes, 4.5 L of packed bovine erythrocytes was washed with 45 L of PBS solution and repacked by centrifugation at 3,000 × *g* and 4°C for 30 minutes. The supernatant and buffy coat were aspirated. Approximately 25% of the total volume of erythrocytes was sacrificed to completely remove the buffy coat. The washed packed erythrocytes were lysed at 4°C during a period of 2 hours by the addition of 5 volumes of cold 0.5mM sodium phosphate (pH, 7.2) containing 1mM EDTA, 0.2% NaN_3, and 0.1mM phenylmethylsulfonyl fluoride.[e] The hemoglobin was removed by rapid cycling in the retentate channel of a cassette system using tangential-flow filtration and equipped with 0.45-µm pore-size filter cassettes.[j] The membranes were washed with excess cold lysis buffer (without the enzyme inhibitor), until the membranes were just slightly pink. The same apparatus was used to concentrate the membranes, which was then stored at –70°C. To remove C3 or C3b binding proteins from the erythrocyte surface, 1.5 mL of packed cells were incubated with 10 mg of trypsin[e] (9,700 units/mg) for 15 minutes at 37°C at a pH of 7.2.[16] The reaction was stopped with soybean trypsin inhibitor, and the cells were washed extensively in PBS solution containing a mixture of protease inhibitors and then with PBS solution alone.[16]

Factor I cofactor activity associated with the bovine erythrocyte surface—Bovine C3b (1 µg) was incubated for 45 minutes at 37°C with various combinations of bovine factors I and H or packed bovine erythrocytes (obtained from 40 µL of a 2% suspension). The buffer used was GBS-EGTA. Normal and trypsinized erythocytes,[16] as well as erythrocyte membranes, were tested for cofactor activity. Prior to use, the erythrocyte samples had been washed in buffer containing a mixture of protease inhibitors. The reaction was stopped by centrifugation at 3,000 × *g* and 4°C for 10 minutes to remove the cells or cell membranes, and the supernatant was analyzed on 7.5% SDS-polyacrylamide gels. Protein concentrations were determined by use of a commercially available protein assay system.[d] Bovine serum albumin standards were prepared fresh for each assay.

Results

Purification of bovine factor I proceeded in a manner similar to that of human factor I, although complete elution of the protein from the cation-exchange column was not achieved with the 0.02 to 0.20M NaCl gradient. The protein continued to trail after the gradient was complete, and a final wash of the column with 1.0M NaCl was necessary to achieve the maximum yield (1 to 2 mg from 1 L of serum) by this procedure. Under nonreducing conditions, electrophoresis of the purified bovine protein revealed that it had an apparent molecular weight of 94 kd; under reducing conditions, the protein separated into 2 chains with molecular weights of 51 and 42 kd (**Fig 1**). The molecular weights of the heavy and light chains of bovine factor I were slightly different from the corresponding human factor I chains.

Degradation of bovine C3b by bovine factor I in

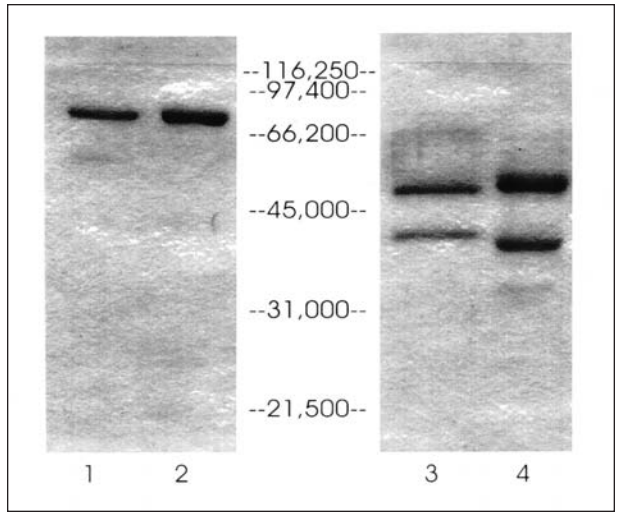

Figure 1—Gel results of SDS-PAGE (10% polyacrylamide with Coomassie blue stain) for analysis of bovine factor I and human factor I under nonreducing and reducing conditions. The samples were as follows: lane 1, nonreduced bovine factor I (1 µg); lane 2, nonreduced human factor I (2.2 µg); lane 3, reduced bovine factor I (1.6 µg); and lane 4, reduced human factor I (3.3 µg). The positions and molecular weights (in Daltons) of SDS-PAGE protein standards are indicated.

the presence of bovine factor H was examined (**Fig 2**). Results of SDS-PAGE analyses indicated that the C3b preparation was composed of an α′-chain (103 kd) and a β-chain (66 kd). A 110-kd component was observed in particular C3b preparations, and this was identified as the α-chain of a small amount of C3 that was present. Neither factors I nor H alone caused cleavage of any of the C3b or C3 chains. In the presence of both bovine factors I and H, the α′-chain of bovine C3b disappeared, as it was cleaved into 2 fragments with apparent molecular weights of 62 and 46 kd. As expected, there was no apparent cleavage of the α-chain of the C3 present in the C3b sample. The degradation fragments of the α′-chain of C3b produced by bovine factor I in the presence of factor H remained covalently linked to the intact β-chain under nonreducing conditions (data not shown). Human factor I produced a similar factor H-dependent cleavage of the bovine C3b α′-chain (Fig 2). With both factor I preparations, there was no apparent fragmentation beyond the iC3b product.

Bovine C3b mixed with bovine erythrocytes, trypsinized bovine erythrocytes (**Fig 3**), or bovine erythrocyte membranes did not undergo any observable fragmentation. The addition of factor I to any of those mixtures resulted in degradation of the α′-chain, and the characteristic band pattern for iC3b on SDS-PAGE (eg, 62- and 46-kd fragments from the α′-chain and the 66-kd β-chain) was observed. Bovine C3b mixed with trypsinized erythrocytes revealed that the cells provided the necessary cofactor activity for factor I cleavage of C3b, comparable to that provided by factor H. Erythocyte membranes had similar characteristics (Fig 3), as did normal erythocytes (data not shown). Binding of C3b-coated microspheres to bovine erythrocytes[17] was unsuccessful (data not shown). Six bovine serum ACP proteins were analyzed via SDS-PAGE (**Fig 4**). Bovine properdin,[18] isolated in its activated form by procedures that yield activated human properdin, was a single polypeptide chain with an apparent molecular weight of 58 kd in the reduced form.

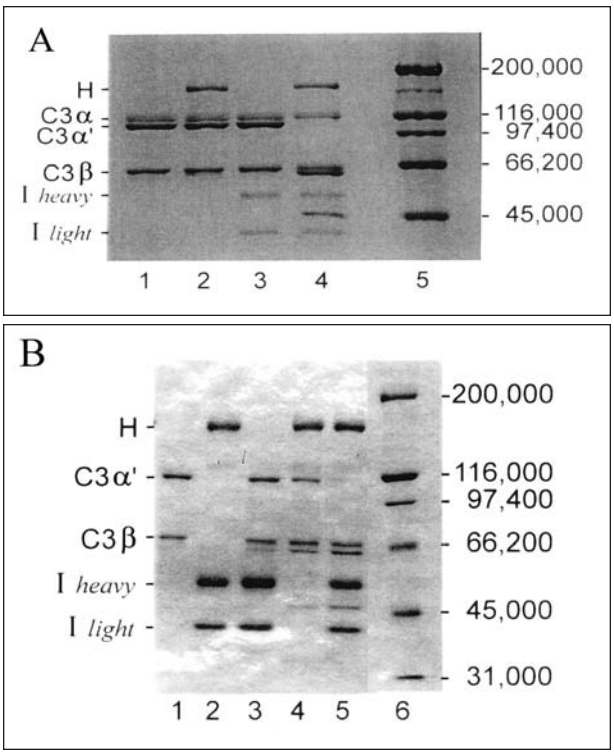

Figure 2—Gel results of SDS-PAGE (10% polyacrylamide, reducing conditions, and Coomassie blue stain) for analysis of bovine factor I-mediated cleavage (A) and human factor I-mediated cleavage (B) of the α′-chain of bovine C3b, with bovine factor H as cofactor. A—Lane 1, bovine C3/C3b; lane 2, bovine C3/C3b plus bovine factor H; lane 3, bovine C3/C3b plus bovine factor I; lane 4, bovine C3/C3b plus bovine factor H plus bovine factor I; and lane 5, molecular weight standards. All samples (1 to 2 μg of each reactant) were incubated at 37°C for 30 minutes prior to analysis. B—Lane 1, bovine C3b; lane 2, human factor I plus bovine factor H; lane 3, bovine C3b plus human factor I; lane 4, bovine C3b plus bovine factor H; lane 5, bovine C3b plus bovine factor H plus bovine factor I; and lane 6, molecular weight standards. All samples (1 to 2 μg of each reactant) were incubated at 37°C for 30 minutes prior to analysis. The positions and molecular weights (in Daltons) of SDS-PAGE protein standards are indicatedin both panels

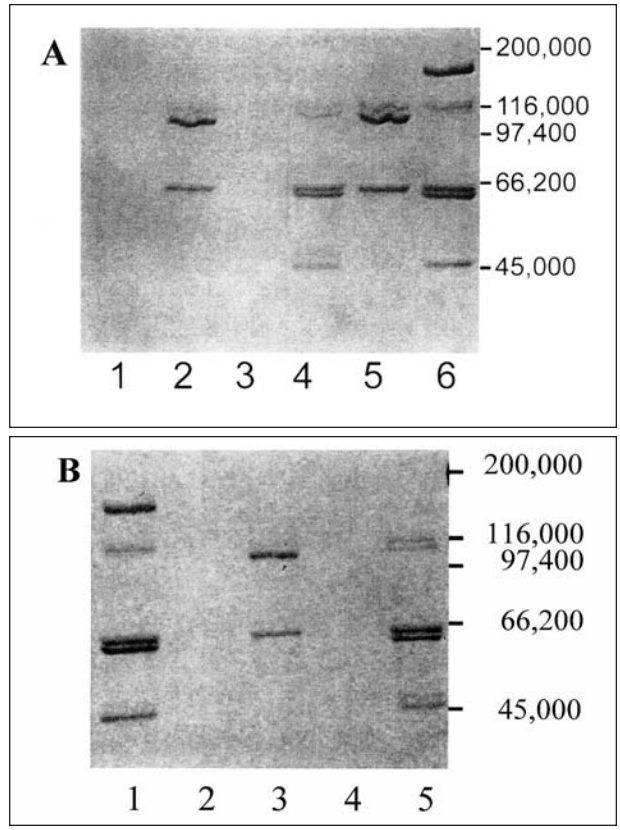

Figure 3—Gel results of SDS-PAGE (7.5% polyacrylamide, reducing conditions, and Coomassie blue stain) for analysis of bovine factor I cofactor activity associated with trypsinized bovine erythrocytes (A) and bovine erythrocyte membranes (B). A—Lane 1, trypsinized bovine erythrocytes; lane 2, trypsinized erythrocytes plus bovine C3b; lane 3, trypsinized erythrocytes plus bovine factor I; lane 4, trypsinized erythrocytes plus C3b plus factor I; lane 5, C3b plus factor I; and lane 6, bovine C3b plus bovine factor I plus bovine factor H. All mixtures were incubated at 37°C for 45 minutes and centrifuged to pack and remove erythrocytes. The amounts of protein used in the reaction mixture were approximately 1 μg C3b, 1 μg factor H, and 0.1 μg factor I. B—Lane 1, bovine C3b plus bovine factor I plus bovine factor H; lane 2, bovine erythrocytes; lane 3, bovine erythrocytes plus bovine C3b; lane 4, bovine erythrocytes plus bovine factor I; and lane 5, bovine erythrocytes plus bovine C3b plus bovine factor I. All mixtures were incubated at 37°C for 45 minutes and centrifuged to remove the cell membranes. The position and molecular weights (in Daltons) of SDS-PAGE protein standards are indicated in both panels

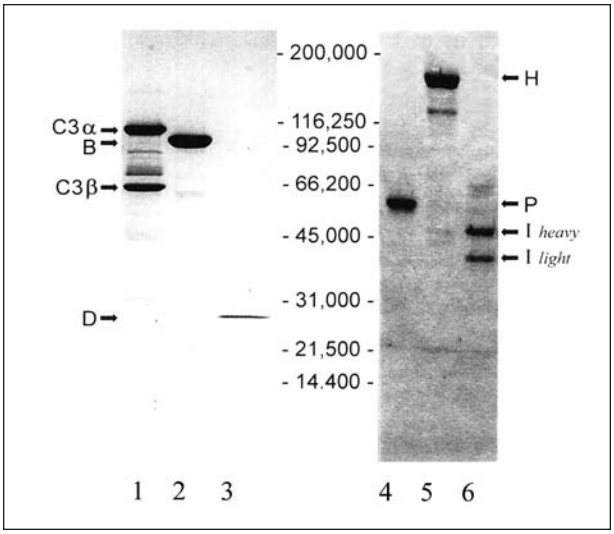

Figure 4—Gel results of SDS-PAGE (5 to 20% polyacrylamide gradient and Coomassie blue stain) for analysis of bovine alternative complement pathway components under reducing conditions. All protein samples (approx 1 mg/mL) were reduced with 2-mercaptoethanol. The samples were as follows: lane 1, C3; lane 2, factor B; lane 3, factor D; lane 4, properdin; lane 5, factor H; and lane 6, factor I. The position and molecular weights (in Daltons) of SDS-PAGE protein standards are indicated.

Discussion

In the study reported here, a bovine serum protein was isolated that had the functional and structural characteristics of the factor I complement control protein detected in other species.[6] Bovine factor H serves as a cofactor for the bovine factor I cleavage of fluid-phase bovine C3b. Although the actual cleavage site was not identified in our investigation, it is an arginine-serine link in the C3b α′-chain in all other mammals and avians.[19] Factor I activity in fetal calf serum, which promotes the cleavage of human C3b in the presence of human or bovine factor H, has been detected in several studies.[20,21] Although human factor I can cleave bovine C3b in the presence of bovine factor H,[10] this combination has no enzymatic effect on the human C3 derivative.[22] It has been noted that homologous combinations of human factors H and I are more effective than heterologous combinations.[23] Physiologic fragmentation of human C3b to iC3b by human factor I in the presence of a cofactor is a 2-step process that results in the excision of a small (2- to 3-kd) fragment (C3f) from the α′-chain of C3b.[6] No evidence to support a 2-step cleavage of bovine C3b by either bovine or human factor I was observed in the study of this report.

Results of the experiments performed in our study to determine whether a membrane-bound cofactor is present on bovine erythrocytes indicated that intact erythrocytes, trypsinized erythrocyes, and membrane ghosts act like factor H in facilitating the conversion of C3b to iC3b by factor I. Trypsinization, which destroys the factor I cofactor activity on primate erythrocytes,[24] does not appear to have altered the presence or activity of the cofactor on bovine erythrocytes. Structural characterization of this membrane-associated activity was not pursued in our study. Complement receptors have been identified (but not well characterized) on bovine eosinophils and macrophages. Bovine neutrophils express cell membrane-associated CR1.[25] The erythrocyte preparations used in the study reported here had no (or negligible) contamination with neutrophils or any other WBCs. On human erythrocytes, CR1 plays an important role in the clearance of immune complexes and regulation of the activity of the classical and alternative pathway C3 and C5 convertase enzymes. There is general agreement that bovine erythrocytes lack CR1-like molecules, and this was supported in our investigations by the inability to demonstrate binding of C3b-coated microspheres to these cells. It is probable that the cofactor activity is related to MCP (CD46), although this factor has not yet been identified on bovine erythrocytes. Sheep erythrocytes express a factor I-dependent cofactor activity that regulates alternative pathway activity.[26] This activity did not cause any accelerated decay, which suggests that it is an MCP-like activity.

Bovine properdin was first described and isolated almost 50 years ago, but it was only briefly studied for its function, and detailed characterization was not performed.[18] In this study, we report that the reduced form of the molecule is a 58-kd polypeptide chain.

In a broad context, results of studies of the complement systems of various species have illustrated the remarkable similarity of this system in animals of diverse lineage. The experimental results of the study reported here clearly confirmed that the isolated bovine C3, factor B, and factor D proteins are the essential complement components required for the generation of the alternative pathway bovine C3-convertase and that isolated factors H and I are effective regulators. The indication that bovine erythrocytes have an inherent cofactor for factor I is intriguing. The biochemical composition of the surface on which bovine C3 convertase is organized modulates the formation and stability of this complex and consequently affects the generation and binding of C3b by that surface. This event is important for immune clearance by phagocytic cells and membrane attack by C5b-9.

[a]Quidel, San Diego, Calif.
[b]QAE-50 Sephadex, Pharmacia Fine Chemicals, Uppsala, Sweden.
[c]CM-Sephadex, Pharmacia Fine Chemicals, Uppsala, Sweden.
[d]Sephacryl S-200, Pharmacia Fine Chemicals, Uppsala, Sweden.
[e]Sigma Chemical Co, St Louis, Mo.
[f]CNBr-Sepharose 4B, Pharmacia Fine Chemicals, Uppsala, Sweden.
[g]Bio-Rad Laboratories Ltd, Richmond, Calif.
[h]Q-Sepharose Fast Flow, Pharmacia Fine Chemicals, Uppsala, Sweden.
[i]Protein-G sepharose, Sigma Chemical Co, St Louis, Mo.
[j]Minitan ultrafiltration system, Millipore Corp, Bedford, Mass.

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