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Reduction and Proteolytic Degradation of Immunoglobulin A from Human Colostrum*

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Human colostrum IgA was split by reducing and proteolytic agents. The product obtained by reduction with β-mercaptoethanol was investigated by urea starch gel electrophoresis and compared with *serum* IgA and *serum* IgG treated in the same way. Heavy and light chains were separated by gel filtration of reduced colostrum IgA and serum IgG. The separated chains were analyzed by urea starch gel electrophoresis at acid and alkaline pH.

After proteolytic degradation a 3.5 S component was isolated from colostrum IgA and compared electrophoretically and immunologically to the 3.5 S fraction from serum IgG. The 3.5 S fraction from the colostrum IgA, in contrast to the corresponding IgG fraction, did not contain any component comparable to the Fc fragment.

The isolation of IgA from human colostrum by column electrophoresis and subsequent gel filtration on Sephadex G-200, yielding an immunoelectrophoretically pure product, was described in a previous communication.[1] The protein was characterized with regard to amino acid composition, N-terminal amino acid residues and sedimentation velocity. In the present work the results of an investigation concerning the properties of the protein after reductive or proteolytic cleavage will be reported.

MATERIALS

Human colostrum IgA (IgA-HC) *** was isolated as described by Axelsson *et al.*[1] The protein was immunoelectrophoretically pure when analyzed with a potent, multivalent antihuman colostrum.[2]

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*** The IgA in colostrum is abbreviated IgA-HC in this paper, while the serum IgA is abbreviated IgA-HS.

Human serum IgA (IgA-HS) was prepared from pooled normal serum by the zinc sulfate precipitation method essentially as described by Heremans.[3] The IgA was further purified by ammonium sulfate precipitation, gel filtration on Sephadex G-200, and column electrophoresis. Preparations which did not contain impurities of IgG or IgM as judged by immunoelectrophoresis with anti-human serum were used. In a few preparations the zinc precipitation was omitted and replaced by ion exchange chromatography on DEAE-Sephadex A-50.[4]

Human serum IgG prepared from a Cohn fraction II was kindly supplied by AB Kabi, Stockholm. The preparation did not contain IgA or IgM.

Antisera. Rabbit anti-colostrum and anti-7S-γ-globulin immune sera were obtained as described previously.[1] Anti-IgA-HS immune serum was obtained from Behringwerke, Marburg a/L, Germany.

METHODS

Reductive cleavage of proteins was performed with 0.1 M β-mercaptoethanol in tris-HCl buffer, pH 8.0 and 8 M urea as described by Edelman and Poulik.[5] The reaction was carried out in a nitrogen atmosphere for 4 h at room temperature. The solutions were then either immediately subjected to starch gel electrophoresis or alkylated with iodoacetamide before the electrophoresis.[5]

Separation of polypeptide chains. Reduction and alkylation in the abscence of urea was performed as described by Fleishman *et al.*[6] Separation of the chains was done by gel filtration in 1 M acetic acid on Sephadex G-150, 3 × 95 cm at + 1 to + 2°. The flow rate was kept at 20 ml/h by means of a peristaltic pump. In some experiments reduced but non-alkylated materials were subjected to gel filtration in deaerated and nitrogen saturated 1 M acetic acid containing 0.01 M β-mercaptoethanol. Applications of samples were performed with the layering technique described by Flodin and Killander.[7] The ultraviolet light absorption at 254 mμ was continuously recorded by a "Uvicord" absorptiometer (LKB, Stockholm, Sweden), and the effluent collected in 10 ml fractions. The optical density at 280 mμ of each 10 ml fraction was also measured. Pooled fractions were concentrated by ultrafiltration through Visking dialysis tubings (8/32) at 4° or lyophilized.

Proteolytic degradations and separation of fragments. Immunoglobulins were degraded with crystalline papain[8] (enzyme-substrate ratio, 1:100) or crystalline trypsin [9] (enzyme-substrate ratio, 1:25). In some experiments with trypsin, cysteine was added to a concentration of 0.01 M. The tryptic degradations were performed in 0.2 M tris-HCl buffer, pH 8.0, for 16 h at 37°.

Separation of degradation products was carried out by gel filtration on Sephadex G-100, using a column of the dimensions 3.5 × 49 cm, equilibrated with 0.02 M tris-HCl buffer, pH 8.0, and 0.2 M NaCl. The filtrations were performed as described above for the separation of polypeptide chains. The flow rate was kept at 12 ml/h.

Thin-layer gel filtration and *thin-layer gel filtration-electrophoresis.* The methods described by Johansson and Rymo [10] and Hanson *et al.*[11] were used.

Ultracentrifugation. Ultracentrifugal analyses were run in a Spinco analytical ultracentrifuge, Model E, at 59 780 rpm and at a constant temperature of 20° ± 0.15°. The experiments were performed with 4° standard cells and wedge cells. Calculations of $s_{20,w}$ were made with an assumed value of 0.72 for the specific partial volumes of the protein material.

Starch gel electrophoresis was performed with the gel in a horizontal position. Hydrolyzed starch from Connaught Medical Laboratory, Toronto, was used for the preparations of the gels. Enzyme-hydrolyzed material was run according to the conditions described by Smithies.[12] For urea-starch electrophoresis of reduced material two different buffers were used: 0.05 M formate buffer, pH 2.8 [5,13] and 0.035 M glycine buffer, pH 8.8.[14] At electrophoresis of non-alkylated products 0.02 M β-mercaptoethanol was included in the buffer. The urea-starch gel electrophoretic experiments were run for 24 h at 3.5 V/cm.

Agar gel electrophoresis was performed mainly as described by Wieme.[15] Agar gels were prepared from 1 % (w/v) suspensions of agar in 0.05 M sodium barbital buffer, pH 8.6. 2 ml of hot agar solution were poured on microscope slides (2.5 × 7.6 cm). After gelification the slides were kept in a humid chamber overnight before use. The

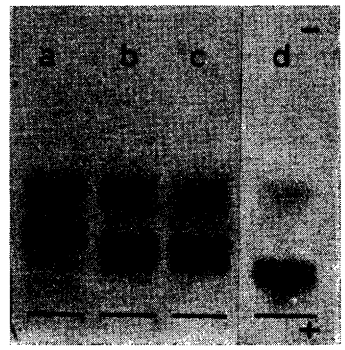

Fig. 1. Starch gel electrophoresis of reduced immunoglobulins performed in a buffer containing 8 M urea, 0.05 M sodium formate, pH 2.8, and 0.02 M β-mercaptoethanol. Duration of electrophoretic run 24 h at 3.5 V/cm. *a*, Serum IgG; *b* and *c*, two preparations of IgA-HC and *d*, IgA-HS.

electrophoretic runs were performed for 24 min at 20 V/cm with petroleum ether (b.p. 60°—85°) for cooling. Fixing, drying and dyeing was carried out exactly as described by Wieme.

Immunoelectrophoresis was made with a slight modification of Scheidegger's method.[16]

Protein determinations were performed with a modification of Lowry's method [17] or a micro Kjeldahl procedure.[18]

N-Terminal amino acid residues were determined by Sanger's method.[19]

RESULTS

The products obtained by reductive cleavage of IgA-HC were separated by urea-starch gel electrophoresis at acid pH and compared with the corre-

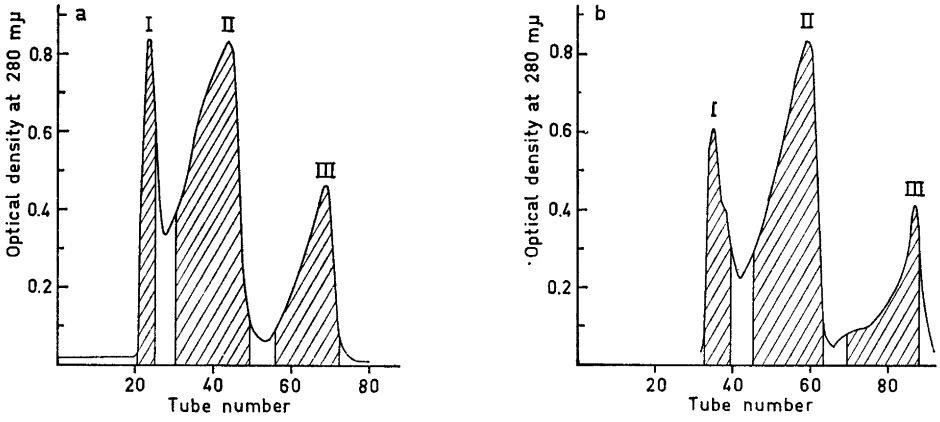

Fig. 2. Gel filtration curves of reduced IgG (*a*) and IgA-HC (*b*) on Sephadex G-150 in deaerated 1 M acetic acid and 0.01 M β-mercaptoethanol. Column dimensions 3 × 95 cm. Flow rate 20 ml/h. *c*. Schematic representation of urea-starch gel electrophoretic patterns at acid pH of fractions obtained from gel filtration of reduced immunoglobulins.

sponding products from IgA-HS and IgG. As seen in Fig. 1 the electrophoretic pattern of the reduced IgG was in accordance with the results obtained by Edelman and Poulik [5] and Fleischman *et al.*[6] with zones corresponding to light chains (L chains), heavy chains (H chains), and some minor fractions behind the heavy chains, which were probably aggregation products. The same principal patterns were shown by the IgA-HC and IgA-HS. The light chains of the three proteins had the same electrophoretic mobility whereas the positions of the heavy chains differed significantly.

Separation of products obtained by reductive cleavage in the absence of urea was performed by gel filtration in 1 M acetic acid. Sephadex G-100, G-150, and G-200 were tested in preliminary experiments. Sephadex G-150 was found most suitable, giving good resolution and acceptable flow rates. In Fig. 2 the gel filtration diagrams of reduced IgA-HC and IgG are shown. The similarity of the curves is clear. The content of protein in each fraction was determined and the results comprised in Table 1. In order

Table 1. Protein-N analyses of gel filtration fractions obtained from separations of reduced IgA-HC and IgG from serum on Sephadex G-150. The values are expressed in per cent of total nitrogen applied.

	Fraction			Per cent recovery of protein-N
	I	II	III	
IgA-HC	14.8	60.1	20.6	95.5
IgG	10.2	64.8	22.8	97.8

to investigate the possible role of alkylation on the products some of the gel filtration experiments were performed by immediate application of reduced but non-alkylated proteins on a Sephadex bed, previously equilibrated with deaerated and nitrogen-saturated acetic acid. No differences were noted between these experiments and those performed with alkylated proteins.

The fractions obtained by gel filtration were concentrated by ultrafiltration through Visking dialysis tubing to a protein concentration of 0.5—1 % and then dialyzed against ice-cold distilled water for two days. Fraction III from some experiments was concentrated by lyophilization instead of ultrafiltration. Fractions I and II gave rather opalescent solutions while fraction III was quite clear. The concentrated fractions were analyzed by urea-starch gel electrophoresis in formate buffer, pH 2.8. The results obtained with IgG indicated the presence of heavy chains (γ-chains) in fraction II and light chains in fraction III. Fraction I contained a major component with approximately the same mobility as the γ-chains and two faint components behind it. Fraction I from IgA-HC analyzed in the same way was shown to contain a single major zone with a faint zone travelling behind, while fraction II contained the two narrow zones observed in the unfractionated material. Fraction III obviously contained light chains. The results are summarized in Fig. 2 *c*.

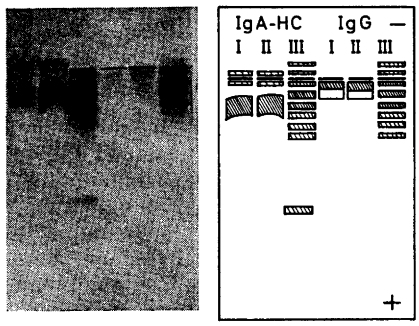

Fig. 3. Urea-starch gel electrophoresis of fractions from gel filtration of reduced IgG and IgA-HC performed in glycine buffer, pH 8.8.

Urea-starch gel electrophoresis of fractions III in glycine buffer, pH 8.8, showed a pronounced heterogeneity (Fig. 3), in accord with the results obtained by Cohen and Porter.[14] The patterns of light chains from IgG and IgA-HC were identical, with the exception of a very fast-moving component occurring only in the case of IgA-HC. Fractions I and II from each protein, when analyzed by this method showed similar patterns, but marked differences were noted between the two proteins (Fig. 3). N-Terminal amino acid determinations of the three gel filtration fractions from IgG and IgA-HC revealed, that aspartic and glutamic acid occupied the N-terminal positions in both the heavy and the light chains of the proteins.

Proteolytic degradation. In preliminary experiments small amounts (samples of 2 mg) of IgA-HC, IgA-HS, and IgG were degraded by trypsin or papain and then analyzed by thin-layer gel filtration on Sephadex G-100. All three degraded proteins contained components moving with the same rate as a reference of serum albumin and evidently corresponding to the 3.5 S component of degraded IgG. The IgA proteins, however, seemed more resistant to proteolytic action than IgG, judging from the large amounts of unretarded material in thin-layer gel filtration. The addition of cysteine at the tryptic degradations considerably increased the amounts of 3.5 S material.

In order to achieve a separation on a larger scale of the enzymatically degraded IgA-HC, column gel filtration on Sephadex G-100 was employed. Three fractions were obtained (Fig. 4), the third of which contained mainly low molecular-weight material. The small fraction obtained from tryptic

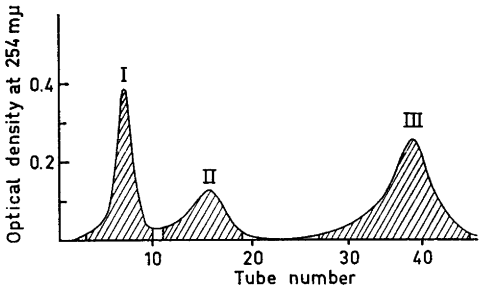

Fig. 4. Gel filtration of a tryptic IgA-HC hydrolysate on Sephadex G-100 in 0.02 M tris-HCl, pH 8.0, and 0.2 M sodium chloride. Column dimensions, 3 × 49 cm. Flow rate 12 ml/h.

hydrolysates of IgG [9] with a sedimentation coefficient of 2 S could not be demonstrated by gel filtration of degraded IgA-HC. Fractions I and II contained about 30 % and 20 %, respectively, of the starting material. These fractions were compared to the corresponding fractions of degraded IgG.

In the ultracentrifuge fraction I from both IgA-HC and IgG was heterogeneous, whereas fraction II showed only one peak (Fig. 5). The sedimentation coefficients for 0.7 % solutions of fractions I and II are given in Table 2.

Table 2. Sedimentation coefficients for 0.7 % solutions of fractions from tryptically degraded IgA-HC and IgG from serum after gel filtration on Sephadex G-100.

Fraction		$s_{20,w}$, 0.7 %
IgA-HC		
	I	12.8; 9.5
	II	3.5
IgG		
	I	6.1; 4.2
	II	3.4
	III	2.0

No separation of fractions I or II from IgA-HC was achieved by agar gel electrophoresis, both fractions showing a single broad component with the same electrophoretic mobility as the undegraded protein, whereas starch

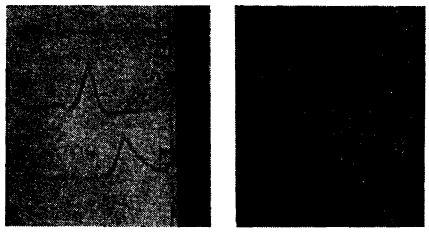

Fig. 5. Ultracentrifugal analyses of fractions obtained by gel filtration of proteolytically degraded IgG and IgA-HC on Sephadex G-100, at protein concentrations of 7 mg/ml. Rotor speed was 59 780 rpm. *a*, IgA-HC fraction I after sedimentation at constant speed for 35 min; *b*, IgG, fraction I after 35 min; *c*, IgA-HC, fraction II after 75 min; *d*, IgG, fraction II after 75 min. Sedimentation from right to left.

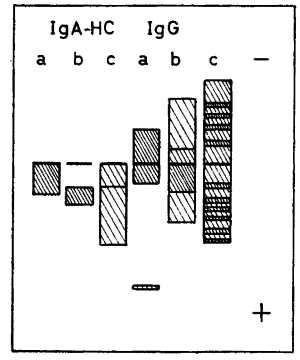

Fig. 6. Starch gel electrophoresis of fractions obtained by gel filtration of proteolytically degraded IgG and IgA-HC on Sephadex G-100, compared with the untreated proteins. *a*, Untreated proteins; *b*, fraction I from Sephadex G-100; *c*, fraction II from Sephadex G-100.

gel electrophoresis revealed differences between the gel filtration fractions and the untreated protein (Fig. 6). The pronounced heterogeneity of the 3.5 S fraction from IgG, owing to the presence of Fc fragments, could not be demonstrated in the 3.5 S fraction from IgA-HC.

Immunoelectrophoretic analyses of proteolytically degraded IgA-HC and IgG are summarized in Fig. 7. The differences in the patterns of degraded

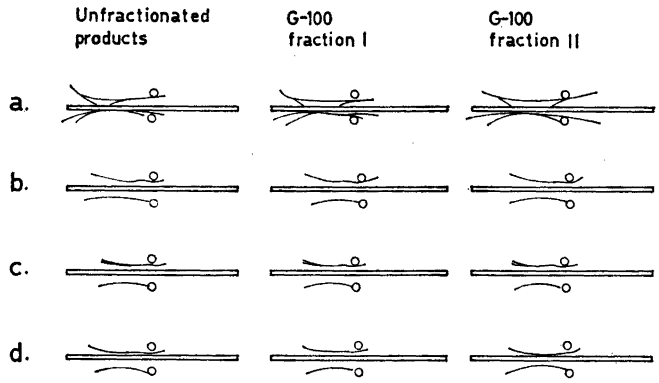

Fig. 7. Immunoelectrophoretic analyses of products obtained by enzymatic degradation of IgG and IgA-HC. The upper basin in each run contains the undegraded protein. *a*, IgG degradation products tested with anti-7S-γ-globulin; *b*—*d*, IgA-HC degradation products tested with anti-7S-γ-globulin (*b*), anti-human colostrum (*c*), and anti-IgA-HS (*d*).

IgA-HC compared to degraded IgG are quite obvious, all products from IgA-HC forming single precipitates with anti-7S-γ-globulin, anti-human colostrum and anti-IgA-HS. Analyses of IgA-HC degraded for 1/2, 1, 2, or 5 h with papain also showed only a single precipitate. The reactions of the gel filtration fractions I and II from IgA-HC with anti-IgA-HS indicate the presence of specific IgA determinants in these fractions. Both fractions have also determinants common to IgG. Tests with a large number of antisera occasionally showed small and faint precipitates in addition to the single line.[20]

DISCUSSION

The use of reductive and proteolytic cleavage in connection with starch gel or urea-starch gel electrophoresis and immunological analyses has provided valuable information on immunoglobulin structure. After reduction of immunoglobulins it is possible to separate two types of polypeptide chains (H and L chains) by urea-starch gel electrophoresis at acid pH.[5] A comparison of reduced IgA-HC, IgA-HS, and IgG, using this method, shows obvious differences in the mobilities of their heavy chains. The differences between the heavy chains of IgA-HC and IgA-HS is hard to evaluate for the present, owing to the difficulties of obtaining representative preparations of normal serum

IgA. The presence of two narrow zones in the presumed heavy chain region of IgA-HC, might indicate an electrophoretic heterogeneity of the heavy chains from IgA-HC, but might also represent an incomplete reduction of the protein or an association tendency of its heavy chains.

The similarities of the light chains prepared from IgA-HC and IgG are well illustrated by their patterns in urea-starch gel electrophoresis at alkaline pH. The fast-moving component, present only in the light chain fraction from IgA-HC, was invariably present at repeated experiments, using different preparations of IgA-HC. The rapid migration of this component in starch gel might indicate a smaller molecular size than the ordinary light chains. The report of van Eijk [21] on the presence of a sub-unit with a molecular weight of 5800 in a Bence-Jones protein is notable in this connection.

Recovery of heavy and light chains from reduced and alkylated IgG and IgA-HC after gel filtration on Sephadex G-150 are in good accordance with the results of Fleischman *et al.*[6] Our results indicate the same proportions of heavy and light chains in the two proteins, provided that the molecular weights of the chains are of approximately the same magnitude.

The studies of proteolytic fragments of IgA by gel filtration indicated the formation of a 3.5 S component, confirmed by sedimentation velocity analyses, whereas no 2 S fraction was found after tryptic degradation as in IgG.[9] In agreement with investigations on IgA from *serum*,[3,22,23] a 3.5 S component was shown to be formed by degradation with papain. The single 3.5 S fragment present in enzymatic hydrolysates of IgA evidently corresponds to the Fab fragment of IgG, whereas the Fc fragment is not demonstrable.[3,22] The lack of fragments corresponding to Fc might depend on differences in the primary structure and the protein conformation in this part of the molecule, which renders it susceptible to attacks of proteolytic enzymes with a break-down to low molecular weight peptides. Such a lability of the Fc fragment to proteolysis with papain and trypsin has been found by Poulik and Shuster [24] in one myeloma protein, type IgG. It might also be noted that chymotrypsin [25] and pepsin [26] have a similar effect on IgG, at least under the experimental conditions reported. The reaction of the 3.5 S fragment with anti-IgA serum is notable, indicating the presence of specific determinants on the Fab-corresponding fragment.

The information hitherto obtained on IgA-HC does not permit the proposal of a structural model for the protein. It is likely that Porter's model, suggested for IgG [27] is also applicable for the IgA-HC, having probably the same proportions of heavy and light chains, but another arrangement of the peptide chains in the IgA-HC cannot be excluded. The presence of the "transport piece" [28] must also be taken into account, when proposing a model for colostrum immunoglobulin A.

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