ISOLATION OF COMPONENTS FROM PEPTIC DIGEST OF NORMAL HUMAN γG IMMUNOGLOBULIN

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The pure $(Fab')_2$ and pFc' fragments were isolated from the peptic digest of normal human γG immunoglobulin. Fragment pFc' was prepared from the fraction which had been obtained by precipitation of the peptic digest with Zn^{2+}-ions to a concentration of 2·5 mM. Fragment $(Fab')_2$ was isolated from the supernatant after precipitation with Zn^{2+}-ions to 25 mM concentration. Both fragments, $(Fab')_2$ and pFc', were purified by gel filtration on Sephadex G 100.

The digestion of γG immunoglobulin with pepsin differs from the digestion with papain in the reaction products. While the digestion with papain (as well as with plasmin or spontaneous digestion) give rise to univalent fragments Fab' and fragment Fc (ref.[1-3]), or alternatively to fragment Fc' (ref.[4,5]), only bivalent fragment $(Fab')_2$ (ref.[6,7]) had originally been identified in the peptic digest. Two additional components were identified recently in the peptic digest of rabbit and human γG immunoglobulin, namely fragment Fab', which represents one half of fragment $(Fab')_2$, and a subfragment of fragment Fc, which has been designated Pep III' and pFc' (ref.[8-10]). The formation of these two fragments (Fab' and pFc') depends on the conditions of the digestion[6,11,12].

This paper describes the isolation of fragments $(Fab')_2$ and pFc' from the peptic digest of normal human γG immunoglobulin and the characterization of the purified preparations by conventional analytical methods.

EXPERIMENTAL

Material and Methods

Human γG immunoglobulin was a lyophilized product supplied by the Institute for Sera and Vaccines, Šarišské Michalany. The immunochemically pure preparation was obtained by purification of the commercial product on DEAE-Sephadex according to Baumstark[13].

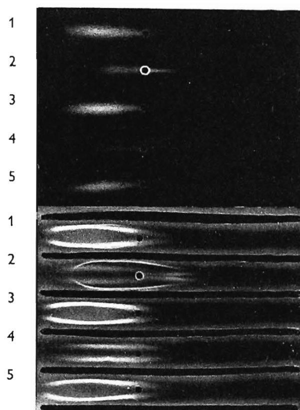

FIG. 1

Agar-gel Electrophoresis and Immunoelectrophoresis of Fractions Obtained by Fractionation of Peptic Digest by Zn^{2+}-ions

1 peptic digest, *2* solution of product precipitated at a concentration of Zn^{2+}-ions not exceeding 2·5 mM, *3* supernatant after precipitation of preceding product, *4* product precipitated at a concentration of Zn^{2+}-ions not exceeding 25 mM, *5* supernatant after precipitation of preceding product. Antiserum SwAHu/IgG-Fab No 8 was used for the precipitation.

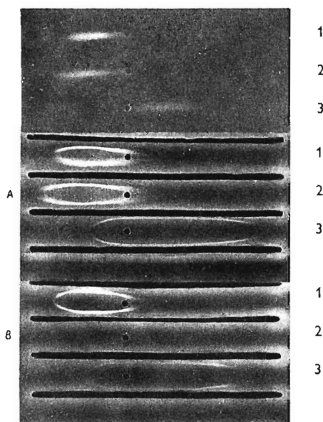

FIG. 4

Agar-gel Electrophoresis and Immunoelectrophoresis of Final Purified Products

1 γG immunoglobulin, *2* fragment $(Fab')_2$, *3* fragment pFc′. *A* precipitation with SwAHu/IgG-Fab No 8, *B* precipitation with SwAHu/Fc + Fc′.

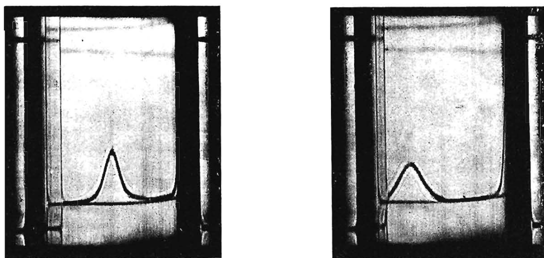

Fig. 5

Sedimentation Analyses of Fragments $(Fab')_2$ (A) and pFc′ (B) of Normal Human γG Immunoglobulin

Sedimentation coefficients ($S_{20,w}$): 5·0 S fragment $(Fab')_2$, 2·4 S fragment pFc′.

Pepsin was a product of Léčiva, Prague and its activity was 1 : 14500 units (determined by titration with casein as substrate). The peptic digestion was effected by 2-hour incubation under the conditions given in the preceding paper[12].

Gel filtration on Sephadex G-100 was carried out on a 1·5 × 80 cm column equilibrated with 0·1M Tris-phosphate buffer containing 0·4M-NaCl, pH 8·0. The fractions were collected at 15 min. intervals at a flow rate of 6 ml per hour and their absorbance at 280 nm was measured.

Immunoelectrophoresis[14] was performed using the micromodification of Škvařil[15]. The antisera employed for the precipitation were the following commercial preparations of Sevac, Prague: unsaturated pig serum to human proteins, containing antibodies to fragments Fab, Fc, and Fc′ (SwAHu/IgG-Fab No. 8) and pig monospecific antiserum to fragments Fc + Fc′ (SwAHu/Fc + + Fc′). The proteins were stained with amido black 10 B. Agar gel electrophoresis was carried out under the same conditions as immunoelectrophoresis.

Sedimentation analysis was performed in Model E Spinco Ultracentrifuge at 52640 r.p.m. in 0·15M-NaCl with a 1% solution of the protein. The sedimentation coefficient was calculated according to Svedberg[16].

Isolation of Fragment pFc′

The pH of the peptic digest was adjusted to pH 7·2 ± 0·1 by 10% NaOH and then precipitated at room temperature with 0·1M zinc acetate until the final concentration of Zn^{2+}-ions was 2·5 mM. After the precipitation had been completed, the pH was adjusted again to pH 7·2, the reaction mixture was stirred one hour and then set aside overnight at +4°C. The isolated precipitate containing mostly fragment pFc′, was washed three-times with 2·5 mM zinc acetate. After the last washing the precipitate was dissolved in 0·5% sodium citrate. (The dissolving was complete and a slightly opalescent solution was formed only after solid citric acid had been added). The solution was dialyzed 48 hours against 0·5% sodium citrate. The dialyzed product was filtered over a layer of K 5 and applied to the Sephadex G-100 column. The samples withdrawn at regular intervals were analyzed immunoelectrophoretically using the antiserum containing antibodies to fragment Fab, Fc, and Fc′. Fractions containing pure fragment pFc′ (cross-hatched area of the graph) were pooled, desalted on a Sephadex G-25 (medium) column, and lyophilized. To obtain an immunochemically pure preparation, the product was subjected to one additional gel filtration over Sephadex G-100 carried out under the same conditions as the first gel filtration.

Isolation of Fragment $(Fab')_2$

The supernatant remaining after the separation of the product precipitated at concentration of Zn^{2+}-ions below 2·5 mM was further precipitated by 0·5M zinc acetate (final concentration of Zn^{2+}-ions 25 mM). The pH was then adjusted to pH 7·2 with 10% NaOH, the mixture was stirred 1 hour and set aside overnight at +4°C. The supernatant, which contained mainly fragment $(Fab')_2$, was separated from the precipitate by centrifugation and dialyzed 48 hours against 0·5% sodium citrate. The dialyzed solution was filtered and applied to the Sephadex G-100 column. Aliquots were analyzed immunoelectrophoretically using precipitation with the antiserum containing antibodies to the determinant groups of fragments Fab, Fc, and Fc′. Fractions containing pure fragment $(Fab')_2$ were pooled, desalted on a Sephadex G-25 (medium) column, and lyophilized. To obtain immunochemically pure fragment $(Fab')_2$, the product was subjected to additional gel filtration over Sephadex G-100 carried out under the same conditions as the first filtration.

RESULTS AND DISCUSSION

The necessary prerequisite of a successful isolation of fragments $(Fab')_2$ and pFc′ from the peptic digest of human γG immunoglobulin is their significantly different behavior during the precipitation with Zn^{2+}-ions. While fragment pFc′ is precipitated at neutral pH at low concentrations of Zn^{2+}-ions, fragment $(Fab')_2$ remains in solution even at higher concentration of Zn^{2+}-ions. The character of individual

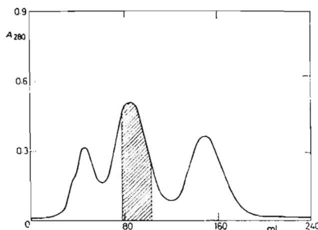

FIG. 2

Elution Profile of Proteins Obtained by Gel Filtration on Sephadex G-100 of Fraction Precipitated at Concentration of Zn^{2+}-ions Not Exceeding 2·5 mM

The position of pure fragment pFc′ is marked in the graph by cross-hatching. *A* absorbance of effluent at 280 nm, ml volume of effluent.

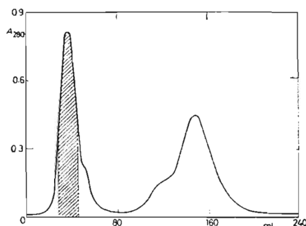

FIG. 3

Gel Filtration on Sephadex G-100 of Supernatant after Separation of Fraction Precipitated at Concentration of Zn^{2+}-ions Not Exceeding 25 mM

Fractions containing pure fragment $(Fab')_2$ are marked by cross-hatching. *A* absorbance of effluent at 280 nm, ml volume of effluent.

fractions of the peptic digest is best seen from their electrophoretic and immunoelectrophoretic behavior shown in Fig. 1*. It is obvious that the predominant portion of fragment pFc' is precipitated at 2·5 mM concentration of Zn^{2+}. The supernatant remaining after separation of this fraction contains as the main protein component fragment $(Fab')_2$ which is contaminated only slightly with a small amount of fragment pFc' and with the residual intact γ-globulin. These contaminants are removed after the concentration of Zn^{2+} has been increased to 25 mM.

The behavior of peptic fragments pFc' and $(Fab')_2$ of normal human γG immunoglobulin during the precipitation with Zn^{2+}-ions indicates that the most suitable starting material for the isolation of pure fragment pFc' is the fraction precipitated at a concentration of Zn^{2+}-ions not exceeding 2·5 mM. Pure fragment $(Fab')_2$ can be prepared best from the supernatant remaining after separation of the fraction precipitated at a concentration of Zn^{2+}-ions not exceeding 25 mM. Both fragments can be obtained in pure state from the described starting materials by gel filtration on Sephadex G-100.

The profile of gel filtration on Sephadex G-100 of the dialyzed fraction, obtained by precipitation of the peptic digest at 2·5 mM concentration of Zn^{2+}-ions, is shown in Fig. 2. The residue of intact γG immunoglobulin and a small portion of fragment $(Fab')_2$ emerge from the column first and constitute the first peak. The second peak contains pure fragment pFc' (cross-hatched area) and the non-precipitating digestion products are present in the third peak.

The elution curve obtained by gel filtration on Sephadex G-100 of the supernatant remaining after precipitation with 25 mM concentration of Zn^{2+}-ions shows two main peaks (Fig. 3). Pure fragment $(Fab')_2$ (cross-hatched area) emerges in the first peak while the low-molecular weight digestion products are eluted in the second peak.

If immunochemically pure fragments $(Fab')_2$ and pFc' are to be obtained, it is necessary to subject the purified preparations to one additional gel filtration. The fragments obtained after repeated gel filtration were analyzed by agar-gel electrophoresis and immunoelectrophoresis using pig antiserum containing antibodies to fragments Fab,Fc, and Fc' (Fig. 4*). The results of these analyses show that the electrophoretic mobility of fragment $(Fab')_2$ is lower than the mobility of intact γG immunoglobulin. Fragment pFc' shows a considerably higher mobility than intact γ-globulin, its mobility, however, is lower than the mobility of analogous fragment Fc' isolated after papain[4] or spontaneous[5] digestion of γG immunoglobulin. Both preparations are immunochemically pure as evidenced also by the results of ultracentrifugation analysis (Fig. 5*). The final preparations give only one peak when analyzed as 1% solutions in 0·15M-NaCl. The sedimentation coefficient calculated for 20°C and distilled water as solvent is 5·0 S for fragment $(Fab')_2$ and 2·4 S for fragment pFc'.

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* See insert on p. 1624

The described procedure of isolation of the two main peptide fragments, $(Fab')_2$ and pFc', of human γG immunoglobulin represents a relatively simple method which can easily be performed in every laboratory since it does not require special equipment. Especially convenient is the isolation of fragment pFc' based on the concentration of the fragment in the product precipitated at a concentration of Zn^{2+}-ions not exceeding 2·5 mM. Fragment Fab' is not contained in this product and therefore the repeated filtration[10] which is necessary when other isolation procedures are employed and which makes the isolation procedure considerably longer and leads to considerable losses of the isolated component, can be omitted.

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