Proteolysis of Rabbit Immunoglobulin M by Papain

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1. Digestion of rabbit immunoglobulin M (IgM) by papain for 5 h, in the absence of reducing agent, gives Fab μ and Fc μ fragments in high yield. Shorter periods give fragment Fc μ with one or more Fab μ fragments still attached. 2. Reducing agent in the absence of a denaturant cleaves rabbit IgM into half-subunits, each containing one μ chain and one light chain. Digestion by papain in the presence of such a reducing agent destroys the Fc μ domains.

The nomenclature used in this paper is that recommended by the World Health Organization (1964, 1969).

To investigate IgM* on rabbit lymphocytic surfaces (Pernis *et al.*, 1970) we are attempting to prepare antisera specific for the Fab μ and Fc μ regions. Preparation of fragment Fc μ is often a problem because of its susceptibility to further degradation. In preparing these fragments from rabbit serum IgM we have discovered an easy and reproducible means of obtaining pentameric fragment Fc μ in good yield.

Digestion of human monoclonal IgM samples by trypsin at elevated temperature or by activated papain has given fragment Fc μ or a mixture of the pentameric and monomeric forms (Plaut & Tomasi, 1970; Onoue *et al.*, 1968; Zikán & Bennett, 1973).

The cleavage of rabbit IgM by proteolytic enzymes is less well characterized. Fragment Fc μ was not identified after digestion of rabbit IgM by papain in the presence of cysteine (Ungar-Waron *et al.*, 1967). We report here that fragment Fc μ can be prepared in good yield from rabbit IgM by digestion with papain pre-activated with dithiothreitol.

Rabbit IgM was prepared from pooled normal serum (Tissue Culture Products Ltd., Slough, Bucks., U.K.). The macroglobulin fraction was selectively precipitated by the addition of polyethylene glycol 6000 (75 g/l; Chesebro & Svehag, 1968). The precipitate obtained after centrifugation (14000g, 20 min at 4°C) was dissolved in 0.1 times the original serum volume of 0.5 M-Tris-HCl–0.1 M-NaCl–0.025 M-EDTA, pH 8.0. A solution of NaCl (153 g/l) and KBr (354 g/l) (density 1.346) was added (250 ml/l). Contaminating low-density lipoprotein was removed by flotation during high-speed centrifugation (99000g, 40 h at 15°C; adapted from Chaplin *et al.*, 1965). The residual IgM-containing gel was resuspended in 0.02 M-Tris-HCl–0.1 M-NaCl–0.001 M-EDTA buffer, pH 8.0, and chromatographed on Sephadex G-150

(5 cm $\times$ 88 cm) in the same buffer. The first peak was eluted immediately after the void volume, at the position expected for IgM and other macroglobulins. This peak was harvested, concentrated and subjected to electrophoresis in a block of Pevikon equilibrated with 0.1 M-Tris-HCl–0.05 M-EDTA, pH 8.0 (Osterland, 1968), to remove α -macroglobulins. A final Sephadex G-200 gel-filtration step in 0.02 M-Tris-HCl–0.1 M-NaCl–0.001 M-EDTA, pH 8.0, ensured the removal of any residual 7S protein. Purified rabbit IgM gave a single symmetrical peak on ultracentrifugal analysis ($s_{20,w}$ = 17.7S at 5 mg/ml; Beckman model E analytical ultracentrifuge), and was antigenically pure as judged by double immunodiffusion on Ouchterlony plates, and by immunoelectrophoresis by using goat antiserum to rabbit whole serum (Hyland Division, Travenol Laboratories Inc., Los Angeles, Calif., U.S.A.; Ouchterlony & Nilsson, 1973). Some IgM preparations showed traces of α_2 -macroglobulin on immunoelectrophoresis by using a sheep anti-(rabbit macroglobulin) serum.

Exposure of rabbit IgM to a reducing agent (10 mg of IgM/ml, 0.01 M-dithiothreitol, pH 8.0; 2 h at 18°C) yields a single species with sedimentation coefficient at infinite dilution of 5.37S. This 5S derivative retained Fab μ and Fc μ determinants and is identified as an IgM half-subunit consisting of one μ and one L chain (Kishimoto & Onoue, 1971).

Monomeric human fragment Fc μ is quickly digested by papain (Mihaesco & Seligmann, 1968), although fragment Fc μ is comparatively stable to the enzyme (Onoue *et al.*, 1968). If the behaviour of rabbit IgM is analogous, the presence of dithiothreitol during digestion would reduce IgM to μ L units, the Fc μ regions of which would be destroyed by the papain. This proved to be the case, since negligible amounts of fragment Fc μ were obtained by digesting rabbit IgM with papain in the presence of 0.01 M-dithiothreitol (enzyme/substrate ratio 1:100, pH 8.0; 16 h at 37°C). Subsequent digestions were carried out in the absence of a thiol reducing agent.

* Abbreviations: IgM, immunoglobulin M (macroglobulin); L, light.

Papain was activated at 17 mg/ml for 15 min at 37°C in 0.1 M-sodium acetate-acetic acid-0.01 M-dithiothreitol-0.002 M-EDTA, pH 5.5. The dithiothreitol was removed by elution through a column (0.5 cm × 8 cm) of Sephadex G-25 (superfine grade) with 0.1 M-sodium acetate-acetic acid-0.002 M-EDTA, pH 5.5. Rabbit IgM was digested with the pre-activated papain (enzyme/substrate ratio 1:10) at 37°C in the above acetate buffer, pH 5.5. The reaction was stopped after 0.25, 1, 5 or 24 h by the addition of iodoacetamide (recrystallized from ethanol; 1.1 mg/mg of papain) for 15 min at room temperature. The fragments were separated on Sephadex G-200. Five elution peaks were obtained (Fig. 1). Peaks D and E consisted of papain, salts and small peptides. The identities of peaks A, B and C were determined by immunoelectrophoresis and immunodiffusion by using sheep antisera to rabbit IgM and to rabbit light

Fig. 1. Sephadex G-200 chromatography of rabbit IgM after papain digestion

The inactivated digests were applied to an upward-flowing column (1.5 cm × 80 cm) of Sephadex G-200. Equilibration and elution were with 0.02 M-Tris-HCl-0.1 M-NaCl-0.001 M-EDTA, pH 8.0. (a) 15 min digest; (b) 1 h digest; (c) 5 h digest. Sedimentation coefficients of peak A material were measured before chromatography by ultracentrifugation of 5 mg/ml digestion mixtures at 20°C,

chains, and guinea-pig antisera to the putative $Fc_5\mu$ and $Fab\mu$ fragments from peaks A and C. The antisera were prepared by using multiple subcutaneous injections in complete Freund's adjuvant.

Sedimentation coefficients of the proteolytic fragments were determined by ultracentrifugation of the digestion mixtures. The sedimentation coefficient of peak A material decreased with digestion time to a value of 11.5S at 5 h, after which there was no change. The peak was symmetrical. This indicated the progressive digestion of 17.7S IgM to yield a fragment identified as $Fc_5\mu$ by four criteria.

First, the sedimentation coefficient is consistent with the expected molecular weight of fragment $Fc_5\mu$ (approx. 300000). Secondly, when the 11.5S fragment was reduced by 0.01 M-dithiothreitol in 0.02 M-Tris-HCl-0.1 M-NaCl-0.001 M-EDTA, pH 8.0, for 2 h at 18°C, a single 3.3S peak was obtained in the analytical ultracentrifuge: this is consistent with the production of monomeric fragment $Fc\mu$, and compares with the 3.2S human fragment $Fc\mu$ prepared by Onoue *et al.* (1968). Thirdly, the fragments that we have identified tentatively as $Fc_5\mu$ and $Fab\mu$ were shown to possess no antigenic determinants in common when examined by immunodiffusion (Plate 1), by using antisera prepared both against whole IgM and against the fragments themselves. In addition to the patterns shown in Plate 1 it was shown that our putative fragment $Fab\mu$, but not fragment $Fc_5\mu$, reacted with antiserum to rabbit light chains. Finally, immunoelectrophoretic analysis of the 11.5S fragment resulted in a single arc, anodic with respect to IgM and fragment $Fab\mu$ by using anti-(rabbit IgM) serum, but no reaction by using an antiserum to the putative $Fab\mu$ fragment.

Peak C material was identified as fragment $Fab\mu$ both immunochemically and on the basis of its sedimentation coefficient of 3.5S. Peak B was not characterized definitively. It gave a line on immunoelectrophoresis with anti-(rabbit IgM) that was much less anionic than fragment $Fc_5\mu$, slightly less anionic than IgM and of similar mobility to fragment $Fab\mu$. It did not appear to contain $Fc\mu$ determinants by immunodiffusion, and it may represent a fragment similar to the fragment $F(ab')_2\mu$ obtained by pepsin digestion of rabbit IgM (Kishimoto & Onoue, 1971).

Digestion of IgM for 0.25 or 1 h (Fig. 1) gave fragments sedimenting at 14.2 and 13.0S respectively. These molecules, intermediate in size between IgM and fragment $Fc_5\mu$, were shown to contain both $Fab\mu$ and $Fc\mu$ determinants by immunoelectrophoresis and immunodiffusion analysis. It appears that the $Fab\mu$ regions of IgM are sequentially cleaved off by papain, so that after 1 h some but not all of the $Fab\mu$ fragments have been removed, whereas after 5 h only fragment $Fc_5\mu$ remains.

Digestions were also carried out for 5 h at pH 6 and 7 (0.1 M- KH_2PO_4 -Na $_2HPO_4$) and these gave analogous results.

EXPLANATION OF PLATE I

Double immunodiffusion analysis of proteolytic fragments of rabbit IgM

(a) Well 1, Protein from peak A, putative $\text{Fc}_5\mu$; well 2, protein from peak C, putative $\text{Fab}\mu$; well 3, sheep anti-(rabbit IgM). There is a reaction of non-identity between the putative $\text{Fc}_5\mu$ and $\text{Fab}\mu$ fragments. (b) Wells 1 and 2, as in (a); well 4, guinea-pig antiserum to peak C; well 5, guinea-pig antiserum to peak A. The complete lack of antigenic identity between the putative $\text{Fc}_5\mu$ and $\text{Fab}\mu$ fragments is confirmed.

Fragment $Fc_5\mu$ from human IgM is composed of the $C_\mu 3$ and $C_\mu 4$ homology regions from each of the ten μ chains (Putnam *et al.*, 1973). Each homology region includes an intrachain disulphide bond enclosing a loop of approximately 60–70 amino acid residues, so that human fragment $Fc_5\mu$ has a total of 20 loops. It is unlikely that rabbit fragment $Fc_5\mu$ differs from human fragment $Fc_5\mu$ by containing the $C_\mu 2$ as well as the $C_\mu 3$ and $C_\mu 4$ homology regions, since the sedimentation coefficients of 3.3S for the monomeric and 11.5S for the pentameric $Fc\mu$ fragments are commensurate with fragments containing 4 and 20 rather than 6 and 30 homology regions respectively.

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