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The effect of limited proteolysis by trypsin and chymotrypsin on bovine colostral IgG1

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Summary. Limited proteolysis of bovine colostral IgG1 by trypsin caused loss of specific antibody activity but column chromatography showed that relatively little cleavage into fragments had occurred. Polyacrylamide-agarose SDS electrophoresis of the 2-mercaptoethanol-treated digest revealed, however, that extensive cleavage of light chains had occurred even though most of the material before reduction had a mol. wt close to that of undigested IgG1. Although a Fab-type fragment was detected in the digest by immunoelectrophoresis it appeared to be only a minor component. Chymotrypsin had little effect upon either the structure or antibody activity of IgG1. These findings may explain the effect of trypsin and chymotrypsin on the bactericidal activity of colostral antibodies.

INTRODUCTION

In the bovine species, colostral IgG1 accounts for the majority of passively-acquired circulating antibody in the suckled newborn calf (Klaus, Bennett & Jones, 1969; Brandon & Lascelles, 1971), and may also contribute to local immunity in the gastrointestinal tract (Logan, Stenhouse, Ormrod and Penhale, 1974). A prerequisite for both these functions is that the IgG1 escapes significant proteolytic digestion in the gastrointestinal tract, yet there is little information available on the effect of the pancreatic proteases trypsin and chymotrypsin on this immunoglobulin. In the preceding paper (Brock, Arzabe, Piñeiro and Olivito, 1976) it was reported that complement-mediated bactericidal activity of naturally-occurring colostral antibodies to *E. coli* 0 111 was principally associated with IgG1, and that incubation with trypsin, but not chymotrypsin, caused a marked reduction of this bactericidal activity without apparently modifying the gel filtration characteristics of colostral IgG1. In this paper the effect of trypsin and chymotrypsin on the structure and activity of purified colostral IgG1 has been investigated, with the aim of elucidating further the mechanism of action of these enzymes on IgG1 in colostral whey.

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MATERIALS AND METHODS

Materials

IgG1 from the colostrum of normal cows or cows immunized with peroxidase was purified by gel filtration and ion-exchange chromatography (A. Piñeiro *et al.*, manuscript in preparation). Trypsin, chymotrypsin, Trasylol, horse-radish peroxidase and rabbit antiserum to IgG1 were as described in the preceding paper (Brock *et al.*, 1976).

Proteolysis of IgG1

IgG1 (2·2 mg in 0·5 ml of 0·05 M Tris-0·02 M $CaCl_2$, pH 7·8) was incubated at 37° with 40 μg of trypsin or chymotrypsin for 6 h. Proteolysis was terminated by addition of Trasylol (4 u/μg enzyme) and the digests examined by immunoelectrophoresis, SDS electrophoresis in polyacrylamide-agarose gel (Shapiro, Viñuela & Maizel, 1967) and by chromatography on a column (90 × 1·5 cm) of acrylamide-agarose AcA44 (L'Industrie Biologique Française, Gennevilliers, France) equilibrated and eluted with 0·05 M Tris, pH 7·8. This gel has a fractionation range of 20,000–200,000 (Boschetti, Tixier & Uriel, 1972).

SDS-polyacrylamide-agarose gels were stained with Coomassie blue and the relative intensities of the various bands estimated by densitometry (TLD 100 Densitometer, Vitatron, Dieren, Holland).

Determination of antiperoxidase activity

IgG1 with antiperoxidase activity was digested with trypsin or chymotrypsin using the same conditions as above, and aliquots (25 μl) removed at intervals and added to 375 μl of 0·05 M Tris-0·15 M NaCl pH 7·8, containing 10 u of Trasylol. Incubation with peroxidase and antiserum to bovine IgG1 was then carried out as described by Brock *et al.*, (1976). Undigested normal IgG1 was used as a control.

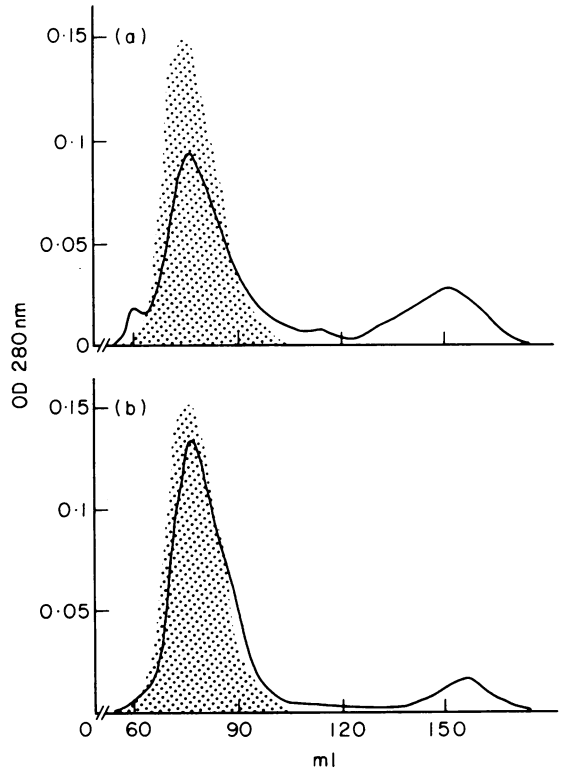

Figure 1. Gel filtration on acrylamide-agarose AcA 44 of (a) trypsin-digested and (b) chymotrypsin-digested bovine colostral IgG1. The shaded areas show the elution position of undigested IgG1.

RESULTS

Column Chromatography of digested colostral IgG1

Fig. 1 shows the patterns of elution from AcA 44 of tryptic and chymotryptic digests of colostral IgG1. In both cases most material eluted in a single peak whose elution volume was very similar to undigested IgG1. This peak accounted for 73 per cent of the material in the tryptic digest and 86 per cent of the chymotryptic digest, virtually all the remainder in both cases being low molecular weight peptides. This suggested that no well-defined fragments had been formed and is in agreement with the gel filtration of IgG1 in colostral whey digested with trypsin or chymotrypsin (Brock *et al.*, 1976).

SDS Electrophoresis

Electrophoresis of the digests and undigested IgG1 without reduction by 2-mercaptoethanol revealed only a single band in each case, though this band was somewhat diffuse in the tryptic digest (Fig. 2). No well-defined fragments were observed. After treatement with 2-mercaptoethanol, undigested IgG1 gave two bands which corresponded to mol. wts of 56,800 and 25,700 and were doubtless due to the heavy and light chains respectively. Additional bands were observed in the reduced proteolytic digests, and in the tryptic digest the band corresponding to light chains was quite weak. Since the heavy chain has a mol. wt approximately twice that of the light chain it would be expected that the intensity of the heavy chain band would be near to twice that of the light chain band in reduced undigested IgG1. For undigested IgG1 the observed ratio was 1·9:1, for the chymotryptic digest it was 1·4:1 and in the tryptic digest 2·7:1, thus indicating preferential cleavage of the light chains in the tryptic digest.

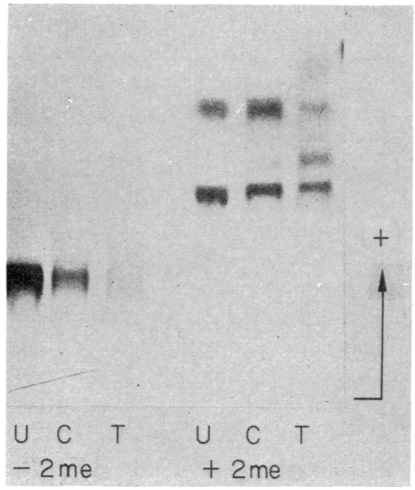

Figure 2. Polyacrylamide-agarose electrophoresis in sodium dodecyl sulphate of trypsin-digested (T), chymotrypsin-digested (C) and undigested (U) bovine colostral IgG1, with (+2 me) and without (−2 me) prior reduction by 2-mercaptoethanol. Stained with Coomassie blue.

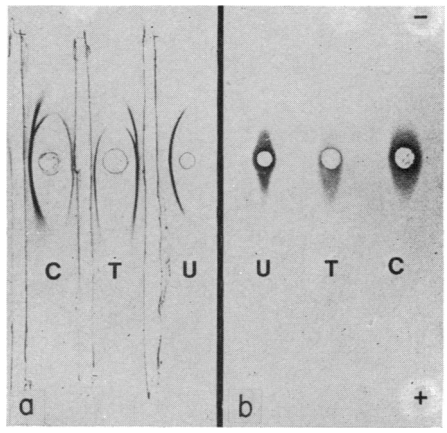

Figure 3. Concurrent immunoelectrophoresis (a) and agarose electrophoresis (b) of trypsin-digested (T), chymotrypsin-digested (C) and undigested (U) bovine colostral IgG1. In (a) the central canal contains rabbit antiserum to IgG1 rendered sub-class specific by absorption with IgG2, and the two outer canals contain unabsorbed IgG1 antiserum. (a) stained with amido black, (b) with Coomassie blue. For further details see text.

Moreover, chain fragments accounted for 53 per cent of the tryptic digest as against only 11 per cent of the chymotryptic digest, this latter being almost entirely accounted for by a band of mol. wt 43,300 (also seen in the tryptic digest) which was presumably a heavy chain fragment. These results indicate that the trypsin digestion had caused much more extensive cleavage of the component chains of IgG1 than had chymotrypsin digestion.

Immunoelectrophoresis

When tested against antiserum to IgG1 that had not been absorbed with IgG2, and therefore retained anti-light chain activity, a major arc and a 'slow' spur were observed (Fig. 3), the latter being suggestive of a Fab type fragment (Fey, 1975). No arcs attributable to Fc-type fragments were observed. When subclass-specific antiserum was used, a single arc appeared, this having in the case of the chymotryptic digest a mobility similar to undigested IgG1, but a slightly 'faster' mobility in the tryptic digest. Since column chromatography and SDS electrophoresis had suggested that no appreciable amount of Fab-type fragments were present in either digest, an electrophoresis was performed in which each sample was applied to duplicate wells, one in each half of the plate, of which one half was then allowed to react with antisera in the normal way and the other stained directly (Fig. 3). The directly-stained portion (Fig. 3b) did not reveal any component in the digests with a more cathodal mobility than undigested IgG1, suggesting that the Fab-type spur derived from a relatively minor component of the digest. In contrast the 'faster' mobility of the IgG1 immunoelectrophoresis arc in the tryptic digest was clearly correlated to a more anodal migration of the directly-stained digest, as compared with chymotrypsin-digested or undigested IgG1.

Antiperoxidase activity

Measurements of loss of antiperoxidase activity during digestion of antiperoxidase IgG1 revealed (Fig. 4) that trypsin caused a steady loss of activity, only about 20 per cent remaining after 24 h digestion, whereas chymotrypsin caused relatively little loss of activity. After 6 h digestion about 55 per cent of the activity remained in the tryptic digest, and about 94 per cent in the chymotryptic digest. If these figures are compared with the percentages of material in the major peak eluting from the AcA

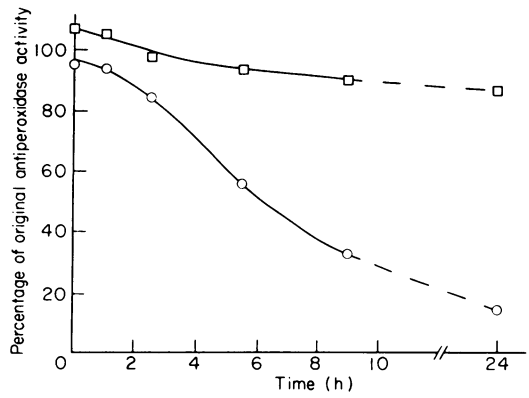

Figure 4. Effect of trypsin (○) and chymotrypsin (□) on antiperoxidase activity of colostral IgG1 from a cow vaccinated with horse-radish peroxidase. Antiperoxidase activity was measured by an immunoenzymatic assay (see text).

chromatography of 6 h digests (73 per cent and 86 per cent respectively) it is evident that the tryptic digest contains material which, though of high mol. wt, has nevertheless lost its specific antibody activity.

DISCUSSION

Although the biological and physico-chemical properties of bovine immunoglobulins have been quite extensively studied, there is less information available on the action of proteases on bovine immunoglobulins than for the corresponding human, rabbit and rodent proteins, even though these enzymes may act on bovine colostral immunoglobulins under physiological conditions in the intestinal tract of the newborn suckling calf.

In the preceding paper (Brock *et al.*, 1976) it was shown that trypsin destroyed the bactericidal activity in bovine colostrum associated with IgG1 more rapidly than chymotrypsin, but that gel filtration of trypsin-treated colostral whey did not reveal any obvious fragmentation of the colostral IgG1. The present work was therefore undertaken primarily to elucidate further the action of trypsin and chymotrypsin on bovine IgG1. Gel filtration of tryptic and chymotryptic digests of purified IgG1 failed, as with colostral whey, to reveal any substantial splitting of the molecule, but SDS electrophoresis in the presence of mercaptoethanol revealed that in the tryptic digest in particular, extensive cleavage of the component chains, particularly of light chains, had occurred. A much lesser degree of cleavage was observed in the chymotryptic digest, which appeared to consist largely of unmodifed IgG1. Since intact light chains are essential for the specific antibody activity of the Fab region, a considerable loss of specific antibody activity would be expected after trypsin digestion, and this was confirmed with the experiments in which the tryptic digestion of antiperoxidase IgG1 was studied. Both tryptic and chymotryptic digests, however, showed bands of Fab-type mobility in immunoelectrophoresis, indicating that some cleavage of the Fc region also occurs, though without detectable release of an intact Fc fragment. The decreased heavy chain to light chain ratio observed in SDS electrophoresis of the reduced chymotryptic digest would accord with a limited attack on the Fc region, although the electrophoresis in Fig. 3b indicates that the amount of Fab-type fragments present is low in both digests.

It thus seems reasonable to postulate two different types of cleavage of bovine IgG1. Firstly, cleavage of the Fab region occurs, particularly of light chains, but the resulting chain fragments are not released, being presumably held together by disulphide bridges. This causes loss of antibody activity, a slight increase in electrophoretic mobility, but no appreciable change in gel filtration characteristics. This is the principal mode of attack of trypsin but does not occur with chymotrypsin. Secondly, the Fc region of the heavy chain is attacked or cleaved yielding an Fab or $(Fab)_2$-type fragment, but no detectable intact Fc. Both enzymes attack IgG1 to a minor extent in this way, and this is the only mode of attack by chymotrypsin, to which IgG1 appears to be quite resistant. These findings could explain the observation (Brock *et al.*, 1976) that tryptic digestion of bovine colostral whey causes loss of IgG1-associated bactericidal activity without apparent cleavage of the molecule, whereas chymotrypsin does not affect the bactericidal activity.

While this paper was being prepared, Fang & Mukkur (1976) published a study of the effect of several proteases, including trypsin, on bovine colostral IgG1, in which they reported that good yields of Fab(t) and Fc(t) fragments were produced. However, their enzyme and substrate concentrations were both more than ten times greater than those

used in the present study, which would doubtless give rise to much greater cleavage. Furthermore, Fang & Mukkur (1976) did not test the biological activity of their Fab(t) fragment, hence it is quite possible that cleavage of the light chains, as reported in the present study, could have occurred prior to, or concurrently with, cleavage of the molecule at the hinge. It does, however, seem probable that the failure to detect Fc in the present study is more likely to be due to the very limited attack on the hinge region, rather than to fragmentation of the Fc region.

From a methodological point of view, it should be noted that some previous studies of *in vivo* proteolysis of bovine immunoglobulins have used gel filtration as the criterion for integrity of structure (Hardy, 1969; Kruse, 1973). The results reported here indicate that this may be unreliable when trypsin is present, and that gel-filtration studies should be complemented with tests of biological activity.

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