Calf rennet lysozyme

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A glycosidase displaying endo-*N*-acetylmuramoylhydrolase specificity (EC 3.2.1.17) was isolated from calf rennet. This lysozyme was also present in abomasal secretions from calf and adult cattle. Multiple molecular forms revealed by electrofocusing might be artefacts. The main enzyme form had M_r approx. 15000, pH optimum 5.0, pI 7.5, and a remarkable conformational stability. Competitive inhibition was observed with both *N*-acetylglucosamine and *N*-acetylmuramic acid, with apparent K_i values of 29 mM and 2.4 mM respectively. The isolated enzyme also displayed significant chitinase activity.

The possible existence of lysozyme in ruminants has long been controversial, in spite of the reported isolation and characterization of a bacteriolytic enzyme present in minute amounts in cow's milk (Shahani *et al.*, 1962; Chandan *et al.*, 1965). Studies on immunoglobulin synthesis in ruminants and on the transfer of passive immunity to their offspring have also demonstrated definite differences between bovine and human milks (Mach & Pahud, 1971). However, secretions such as saliva, tears, nasal, genital or gastrointestinal fluids exert an essentially local protection in both species, which is associated with the presence of secretory immunoglobulins and bacteriolytic enzymes. This prompted us to investigate the possible occurrence of lysozyme in bovine secretions other than milk. No lysozyme activity could be detected in colostral whey, milk whey, saliva or nasal secretions (at their physiological concentrations), whereas a strong lytic activity against *Micrococcus luteus* cell-wall fragments was present in rennet from calf abomasum. We now report the purification and characterization of the glycosidase associated with this bacteriolytic activity. A preliminary report was abstracted in the Proceedings of the 2nd International Symposium on the Ruminant Immune System (Pahud *et al.*, 1981).

Materials and methods

Calf rennet

Crude rennet from calf abomasum (pH 5.4; con-

taining 45 g of NaCl/litre of extract and 1% sodium benzoate) was obtained from the Laboratoire Granday, F-21201 Beaune, France. This extract could be stored for months at +4°C or –20°C without significant loss of lysozymic activity. Calf abomasum and fresh abomasal fluid were provided by a local slaughterhouse.

Purification of enzyme

The purification steps were as follows. (1) Dialysis (36 h) of crude rennet against 10 mM-Tris/HCl buffer (pH 7.0). (2) Anion-exchange chromatography on DEAE-Sephacel (Pharmacia) in the same buffer (column dimensions 26 mm × 400 mm). Under the given conditions, the anion-exchange gel did not adsorb lysozyme, but retained considerable amounts of other proteins and enzymes (e.g. proteinases). (3) Molecular sieving on Sephadex G-75 or G-100 (Pharmacia) in CTS buffer (10 mM-citric acid/30 mM-Tris/0.15 M-NaCl) (column dimensions 15 mm × 900 mm). (4) Electrofocusing on granulated gel (LKB Multiphor) in a pH 4.5–8.5 gradient, according to the manufacturer's instructions.

Enzyme activity measurements

Stable suspensions of *M. luteus* cell-wall fragments (300 µg/ml, unless otherwise indicated) were prepared with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) and were used as a macromolecular substrate, essentially as described by Jollès (1961). Lysis of the bacterial cell fragments was monitored turbidimetrically at 450 nm and 25°C (Shugar, 1952) with a Unicam SP.8-100 spectrophotometer. An absorbance decrease of 1.0/min was arbitrarily defined as one unit of

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lysozyme activity (3 ml cuvettes). The experiments were performed in 0.1 M-sodium acetate buffer (pH 5.0), except for kinetic determinations (in 0.1 M-citrate/phosphate buffer, pH 5.0, unless otherwise indicated). Active fractions were identified at each purification step by the lysoplate technique (Osserman & Lawlor, 1966). Purified peptidoglycan (Park & Hancock, 1960) was used as the substrate in the investigation of enzyme specificity. Amino or aldehyde groups possibly released by enzymic action were detected by standard procedures (Habeeb, 1966; Park & Johnson, 1949). Hexosamine analysis (Fauconnet & Rochemont, 1978) was carried out with a Multichrome B Beckman Analyzer, after reduction (with 25 mM- NaBH_4) and acid hydrolysis (in 4.2 M-HCl for 8 h at 105°C) of the enzymic reaction products. Chitinase activity was detected by the titration of reducing groups (Park & Johnson, 1949) released from colloidal chitin (Jeuniaux, 1966). The synthetic substrates *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-glucopyranoside, *p*-nitrophenyl 2-acetamido-2-deoxy- β -D-galactopyranoside (Koch-Light, Colnbrook, Bucks., U.K.) and *N*-acetyl- β -D-glucosamine naphthol AS-LC (Sigma, St. Louis, MO, U.S.A.) were used to detect non-lysozymic β -hexosaminidase activities in the course of purification. The substrates were dissolved in dimethyl sulphoxide (5% of the final volume) before dilution to the proper concentration (1–10 mM) with the reaction buffer. Release of *p*-nitrophenol from the *p*-nitrophenyl glycosides was measured at 405 nm after alkalization with 0.2 M-sodium borate buffer, pH 9.8 (Li & Li, 1970). The naphthol residues cleaved from the glucosamine derivative were determined at 540 nm after coupling to Diazo Blue B (O'Neil *et al.*, 1979).

Miscellaneous

The K_m value for *M. luteus* cell-wall fragments was determined by double-reciprocal plots (least-squares fits). The K_i values for the inhibitors

N-acetylglucosamine (Fluka, Buchs, Switzerland) and *N*-acetylmuramic acid (Pfanstiehl, Waukegan, IL, U.S.A.) were obtained by the method of Dixon & Webb (1979, pp. 350–351). Rabbit antisera were raised against the purified lysozyme (form D; see below). Each animal was injected with 100 μ g of protein in complete Freund's adjuvant, every 2 weeks for 6 weeks. Immunoelectrophoresis was carried out in 1% agar and 25 mM-barbital (pH 8.3) (Scheidegger, 1955). The protein contents of the various solutions were estimated with Coomassie Brilliant Blue G-250, by the Bio-Rad Laboratories (1979) protein assay.

Results and discussion

Purification

Table 1 summarizes the purification procedure (all steps were at 4°C, and the lytic activities reported here were detected with the macromolecular substrate). The removal of an endogenous inhibitor and/or the significant lowering of the ionic strength (Davies *et al.*, 1969; Saint-Blancard *et al.*, 1970) might explain why the total lysozyme activity was dramatically increased by the dialysis step. Most of the activity was not adsorbed on the anion-exchange gel, which is indicative of a near-neutral pI value. Both β -N-acetylhexosaminidase and lysozyme activities were present in the crude extract and in the anion-exchange eluate. Gel filtration on calibrated Sephadex G-75 or G-100 separated the two entities, which were characterized by relative molecular masses (M_r) of approx. 80 000 and 15 000 respectively (the elution behaviour of the smaller enzyme was not significantly different from that of egg-white lysozyme). Five molecular forms were revealed for the M_r -15 000 species by electrofocusing (peaks A–E in Fig. 1), with pI values of 6.2, 6.6, 7.0, 7.5 and 7.9. The proteinases present in the crude rennet might be responsible for this multiplicity. The main enzyme forms D and E are reported in Table 1, but only form D was char-

Table 1. Purification of calf rennet lysozyme

Purification step	Total protein (mg)	Activity		Purification factor (-fold)	Recovery (%)
		Total (units)	Specific (units/mg)		
Crude extract	1400	345	0.25	—	24
1. Dialysis residue	1320	1410	1.07	4.3	100
2. DEAE-Sephacel eluate	180	1390	7.72	31	99
3. Sephadex G-100 eluate	75	1120	14.9	60	79
4. Isoelectrofocusing (Fig. 1)					
Peak D	43	610	14.2	57	43
Peak E	13	97	7.46	30	7

Fig. 1. Separation of five lysozymic molecular forms by electrofocusing

For details see the text. Fraction size was 5 ml. Maximal lytic activity was arbitrarily set at 100%. Form D represents more than 80% of the total lytic activity recovered at this stage.

Fig. 2. pH-activity profile of calf rennet lysozyme

The kinetic determinations were performed on the macromolecular substrate in 0.1M-citrate/phosphate buffer. The optimum reaction rate was arbitrarily set at 100%.

acterized further. Hen's-egg-white lysozyme (Serva, Heidelberg, Germany), when tested with the macromolecular substrate at pH 5.0 (under the conditions described above) and at optimum pH 9.2 (Davies *et al.*, 1969), was characterized by specific activities of 3.9 and 8.1 units/mg respectively. These values are comparable with the results obtained here for peaks D and E. We consistently obtained adequately linear progress curves (macromolecular substrate), but downward-bending enzyme-concentration curves. Among various possible causes (Dixon & Webb, 1979, pp. 48-55), a limitation in the capacity of the method of reaction estimation might explain this departure from linearity. This problem was minimized by adjusting the reaction rate (absorbance decrease) to 0.1/min or less. All enzyme forms (A-E in Fig. 1) developed sharp precipitation arcs in the β - γ electrophoretic regions when diffused against rabbit antiserum. This is indicative of close structural relationship, in agreement with the hypothesis of the multiple forms being artefacts (resulting from limited proteolysis).

Reaction characteristics

Hydrolysis of soluble peptidoglycan by the purified enzyme released reducing groups, but no

free amino groups. This ruled out any peptidase activity, and on the contrary demonstrated true glycolytic activity. Reduction by NaBH_4 of the enzymically released aldehyde groups caused the disappearance of *N*-acetylmuramic acid (which was converted into muramicitol; Hara & Matsushima, 1966), whereas the *N*-acetylglucosamine moiety remained unaltered. The glycosidase activity can therefore be more specifically defined as an endo-*N*-acetylmuramoylhydrolase activity.

The enzyme is characterized by a sharp pH-activity profile, with less than 1% of optimum activity at neutrality (Fig. 2). This profile might be affected by ionic strength (Davies *et al.*, 1969; Saint-Blancard *et al.*, 1970). When determined at the optimum pH, double-reciprocal plots were adequately linear and yielded a K_m value of $63 \mu\text{g/ml}$. Inhibition by *N*-acetylglucosamine was essentially competitive, with a K_i value of 29 mM. By contrast, the effect of *N*-acetylmuramic acid is susceptible of multiple interpretations. Competitive inhibition was observed (apparent K_i 2.4 mM) in kinetic experiments with inhibitor solutions that had been left at 25°C for at least 3 h. With fresh solutions, an apparent activation was observed (up

to 40%), which gradually disappeared (no effect after approx. 90 min), and was followed by the progressive development of the above-mentioned inhibition. We observed that mutarotation at 25°C and pH 5.0 is comparatively rapid for *N*-acetylglucosamine (equilibrium is reached within 1 h), and slow for *N*-acetylmuramic acid (3–4 h). In addition, the specificity of lysozymes suggests that the configuration of the anomeric carbon atom of *N*-acetylglucosamine is irrelevant for the interaction of the glycoside with the catalytic site, whereas the opposite situation might occur for *N*-acetylmuramic acid. One can therefore rationalize why the effect of *N*-acetylglucosamine did not change with time. By contrast, the two anomeric forms of *N*-acetylmuramic acid might not be equivalent with regard to their action on the enzyme. Indeed, this would explain why the observed global effect varied with time until the mutarotation equilibrium was reached.

Significant chitinase activity was also observed at pH 5.0 for the purified enzyme, as demonstrated by the appearance of aldehyde groups on hydrolysis of colloidal chitin.

Conformational stability

The purified lysozyme preparation was unaffected by 10 min incubations at temperatures in the range 20–70°C (pH 5.0). A similar incubation at 80°C resulted in a 30% loss of activity. Remarkable stability was also observed at pH 2–11. After 4 h incubation at 25°C, no significant deactivation was observed. The effects of freezing, thawing and freeze-drying were also negligible.

The bacteriolytic enzyme described above was first purified from a material obtained by maceration (rennet), but was also found to be the major protein present in the gastric juice from unfed calves and adult cattle (we similarly detected homologous abomasal lysozymes in goats and sheep). In addition, immunofluorescence microscopy on various segments of the digestive tract showed that the detected lysozyme is exclusively secreted by the abomasum (D. Schellenberg, unpublished work). The reactional and physiological properties of the purified enzyme essentially conform to the criteria defined by Jollès (1966) for lysozymes discovered in various species, although its pH optimum and pI values do not coincide with those reported for bovine

milk lysozyme (Chandan *et al.*, 1965). The concomitant presence of β -*N*-acetylhexosaminidase and lysozyme in abomasal fluid must have physiological implications, which have yet to be defined.

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