An Inhibitor of Phospholipase D in Saliva

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1. Bovine, dog and human saliva contain substances which inhibit the soluble phospholipase D present in grass leaf or celery stalk. 2. The inhibitor in bovine saliva is of high molecular weight and exhibits considerable stability to heat, acids and alkalis. 3. The inhibitor has been purified free from salivary mucoprotein. 4. It is suggested that the inhibitor could protect the upper alimentary tract of a herbage-eating animal from the necrotic action of phospholipase D.

Phospholipase D (EC 3.1.4.4), an enzyme that removes the base from nitrogen-containing phosphoglycerides leaving phosphatidic acid, occurs at very variable amounts in the tissues of higher plants (Quarles & Dawson, 1969). We have now found that Perennial Rye (S24) or mixed pasture grasses contain activities which hydrolyse 1.1–2.1 μg of choline/min per mg wet wt. of grass from a phosphatidylcholine substrate when assayed by a standard technique (Dawson & Hemington, 1967). Although the degree of activity is no greater than that found in many green leaves (Quarles & Dawson, 1969) the amount of enzyme ingested by an animal such as a ruminant existing entirely on a grass diet must be considerable. We carried out an analysis of the phospholipids in the oesophageal boli of cattle eating grass, the bolus being caught by hand through a rumen fistula on delivery to the rumen (Reid *et al.*, 1962). This analysis gave strong evidence for a diminution in phospholipase D activity (Dawson & Hemington, 1974).

It is now shown that bovine saliva, as well as that from other species, has a distinct inhibitory action towards phospholipase D. Here we describe the inhibitor involved and its partial purification.

Materials and Methods

Collection of saliva

Bovine saliva was collected with a perforated suction tube placed under the tongue while the animal was eating and held in position with a harness. Particulate matter was removed by a light centrifugation (3 min at 1000g). Human saliva was obtained from donors. Dog saliva was obtained from a 'Boxer' salivating in response to tempting with chocolate.

Phospholipase D

The method of assay has been described by using ovophosphatidylcholine as substrate and measuring

choline release (Quarles & Dawson, 1969). On some occasions the liberation of phosphatidic acid was determined. Here the incubation was stopped by addition of 6 ml of chloroform-methanol (2:1, v/v), shaking well and centrifuging to separate the phases. One-quarter of the lower chloroform-rich layer was quantitatively spotted for separation of the phospholipids by t.l.c. (Silica gel H; chloroform-methanol-water-acetic acid; 65:50:4:1, by vol.). The phosphatidic acid (identified by the use of markers) was located and its phosphorus content determined by methods already described (Scott & Dawson, 1968).

The source of enzyme was a supernatant obtained by centrifuging (100 000g for 1 h) an homogenate prepared by disintegrating 1 g of grass leaves or celery stalk in 10 ml of water (Ultra-Turrax homogenizer). The incubation conditions were, unless otherwise stated, 30°C for 20 min.

Phospholipase A₂ and C

Bacterial phospholipase C was assayed by dispersing 120 μg of ovophosphatidylcholine P in 1.2 ml of 0.0125 M-dimethylglutaric acid buffer, pH 6.8, containing 17 mM-CaCl₂ and incubating for 20 min at 30°C with 5 μg of enzyme [*Clostridium perfringens* phospholipase C; Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.]. Water-soluble P_i liberated was determined as described by Bangham & Dawson (1962).

Venom phospholipase A₂ was measured by dispersing 58 μg of ovophosphatidylcholine P in 1.2 ml of 0.0125 M-dimethylglutaric acid buffer, pH 6.8, containing 8 mM-CaCl₂ and incubating for 30 min at 37°C with 25 μg of cobra venom (*Naja naja*, Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.). At the end of the incubation the mixture was shaken with 6 ml of chloroform-methanol (2:1, v/v) and, after centrifugation (1000g, 10 min), a 1 ml sample of the lower phase was dried down *in vacuo* and spotted for t.l.c. (Silica gel H; chloroform-methanol-acetic acid-

water; 65:50:1:4, by vol.). The lysophosphatidylcholine produced (identified by the use of markers) was located and its phosphorus content determined by methods previously described (Scott & Dawson, 1968).

Results

Amount and location of phospholipase D activity in grass

Some 16 samples of mixed pasture grass showed phospholipase D activities which ranged between 1.1–2.1 μg of choline liberated/min per mg wet wt. when tested under the standard conditions described. No significant difference was observed between samples obtained during summer and winter months. Perennial rye grass (Aberystwyth strain S24) grown under artificial light (Grow-Lux fluorescent tubes, continuous) showed an amount of activity in its leaves 4 days after germination of 0.66 μg of choline liberated/min per mg wet wt., which had increased to a constant value of around 1.2 μg /min per mg by 16 days.

The activity was found to be almost exclusively associated with the supernatant fraction when a grass homogenate was centrifuged at 100000g for 1 h. This contrasts markedly with leaves from spinach, cabbage and sugar beet where the enzyme is substantially membrane-bound and was centrifuged down with the plastid fraction (Kates, 1954). However, a soluble fraction of the enzyme has been described in cabbage leaves (Davidson & Long, 1958).

Inhibition of soluble phospholipase D by saliva

Bovine saliva (1.8–2.5 mg of protein/ml) in tenfold dilution consistently produced a substantial (approx. 50%) inhibition of the phospholipase D of a grass supernatant fraction as measured by the choline liberation from phosphatidylcholine (see above). This was confirmed by measurements of phosphatidic acid produced in the system, whose formation was inhibited to an equivalent extent. No other phospholipid apart from phosphatidic acid was formed.

The extent of the inhibition was not altered by a 30 min preincubation of the system in the absence of either substrate or enzyme. Doubling the CaCl_2 present in the incubation medium (25 to 50 mM) had no effect on the inhibition.

Fig. 1 shows the relationship between enzymic activity and saliva concentration. Sigmoidal shaped curves were consistently obtained with a small concentration lag before the inhibition became effective. Dog saliva was the most effective inhibitor. Bovine saliva, which was the most extensively investigated, gave an inhibition of 90% with a range of 15–20%

Fig. 1. *Inhibition of soluble phospholipase D by saliva*

A supernatant prepared by high-speed centrifugation of an aqueous homogenate of grass or celery stalk was used as enzyme. The choline liberated from a dispersed phosphatidylcholine substrate after 20 min incubation at 30°C was measured in the absence and presence of various concentrations of saliva. ●, Dog saliva and grass enzyme, representative of three experiments; ○, bovine saliva and grass enzyme, representative of six experiments; △, human saliva and celery enzyme, representative of two experiments.

v/v, of saliva for samples of saliva collected at different times. Human saliva tested against celery enzyme was the least effective in terms of inhibitory capacity and a long concentration lag was apparent compared with the other species. It is unwise, however, to read too much into such apparent species differences because of the different conditions under which the saliva was collected. The saliva is a mixed secretion of the parotid, submaxillary and sublingual glands and the contribution of these can vary depending on the method of collection and whether food is being ingested at the time.

Properties of the salivary inhibitor

The inhibitor was non-dialysable when bovine saliva was introduced into a sac and dialysed for 1–3 days against 500 vol. of water at 0°C. The inhibitory activity was completely recovered when bovine saliva

was heated for 2 h at 100°C, followed by dialysis or high-speed centrifugation and over 90% was recovered after heating for 4 h at the same temperature. No significant loss occurred on heating at 115°C for 1 h in an autoclave. The inhibitor was also relatively stable to heating with acids or alkali. On admixture with 10% (v/v) of 1M-NaOH 90% of the inhibitory activity survived after 1 h at 70°C, but it was totally lost on heating for 30 min with 50% (v/v) of 1M-NaOH at 100°C. Bovine saliva could be heated for 7 h at 70°C with 5% (v/v) of 2M-HCl with retention of over 90% of the inhibitory activity. All of these samples were dialysed extensively before testing to free them from acid or alkali.

Distinction between inhibitor and salivary mucoprotein and partial purification of inhibitor

Salivary mucoprotein is known to have considerable stability to heat. A sample of the mucoprotein prepared by the method of Lytleton (1964) was inhibitory to grass phospholipase D but on a protein basis less so than whole saliva. This preparation depends on a precipitation reaction of the mucoproteins with cetyltrimethylammonium bromide, and the latter is only removed with difficulty from the complex formed. This cationic detergent is highly inhibitory to cabbage phospholipase D (Dawson & Hemington, 1967), and additional experiments showed this to be true for the present enzyme preparation from grass. The inhibitory action of the mucoprotein preparation could have therefore been due to residual cetyltrimethylammonium ions contained in the preparation.

When bovine saliva was mixed with 5% (v/v) of 2M-HCl and heated at 70°C, the mucoprotein was decomposed with the liberation of free sialic acid as determined by the method of Warren (1959). After 5 h the liberation of sialic acid was maximal but, nevertheless, even after 7 h of such acid hydrolysis, over 90% of the inhibitory activity could be recovered after dialysis of the acid. This indicated that the inhibitor was not mucoprotein unless the acid-hydrolysed residue was itself active.

In further experiments 20 ml of bovine saliva was freeze-dried and then taken up in 2 ml of 0.05M-Tris buffer, pH 7.4. After centrifuging, the supernatant was applied to a column (110 cm × 1 cm) of Sephadex G-200 and eluted with 0.05M-Tris-HCl buffer, pH 7.4. In three runs the peak showing inhibitory activity was clearly separated from the mucoprotein peak, which being of very high molecular weight was not absorbed on to the column (Fig. 2). When the inhibitor peaks from two columns were combined and run on another column (110 cm × 1 cm) of Sephadex G-200 the resulting inhibitor peak obtained represented a 25-fold purification over the original saliva based on protein content (E_{280} and E_{260} determinations).

Action against other phospholipases

Bovine saliva even at 33% (v/v) concentration produced no inhibition of snake venom phospholipase A_2 . Phospholipase C from *Cl. perfringens* was inhibited to the extent of 20% by this same concentration of saliva which would be sufficient to almost completely inhibit phospholipase D.

Fig. 2. Fractionation of bovine saliva on Sephadex G-200

Bovine saliva (20 ml) was freeze-dried to near dryness and the residue made up to 2 ml in 0.05M-Tris-HCl buffer, pH 7.4. The solution was introduced on to a column (110 cm × 1 cm) of Sephadex G-200 and the components were eluted at room temperature with 0.05M-Tris-HCl buffer, pH 7.4. Fractions of volume 5 ml were collected, dialysed overnight against water at 4° and assayed for protein, inhibitory activity and mucoprotein. The curves given are representative of four separate runs. ○, Protein (E_{280}); ●, inhibition of phospholipase D (0.6 ml of fraction); □, mucoprotein (determined as sialic acid released from 0.15 ml on addition of 0.05 ml of 0.4M-HCl and heating for 1 h at 100°C).

Discussion

The most remarkable property of the inhibitory substance in saliva is its stability to heat and both acid and alkaline conditions. The inability of the substance to pass through the membranes of a dialysis sac suggests a macromolecular component, and its behaviour on a column of Sephadex G-200 would indicate a molecular weight of over 100 000 if it could be equated to a globular protein. If the substance were proteinaceous one would expect a protein of such a high molecular weight to be completely denatured by, for example, boiling for 4 h or autoclaving for 1 h at 115°C. However, mucoprotein, the main protein component of saliva, does not precipitate on heat treatment presumably because its high sialic acid content keeps the protein mostly soluble even though denaturation may have occurred. It seemed therefore possible that the inhibitory activity could have been retained on the heat-treated mucoprotein molecule. The results however clearly indicate that the inhibitor cannot be equated to the bulk of the mucoprotein component. Thus the inhibitor is much more stable to acid hydrolysis and it can be completely separated from the mucoprotein by gel filtration. As to whether the substance is a protein must await isolation of the pure inhibitor and its characterization. Preliminary results would suggest that the impure inhibitor is not susceptible to trypsin digestion.

Although the nature of heat-stable macromolecular inhibitor of phospholipase D in saliva remains to be determined it is possible to speculate as to its possible physiological function. Phospholipases are generally powerful necrotic agents and act on lipoproteins, both soluble and in membrane structures. Phospholipase D is no exception (Condrea *et al.*,

1964; Heller & Arad, 1970), and it is possible that the large amounts of soluble enzyme released by mastication of herbage could have a deleterious effect on the epithelium cells of the early alimentary tract unless saliva exerted a protective action. It is generally accepted that a cow chewing grass or other succulent green food adds about 1 ml of saliva/g of leaf (Bailey, 1961), a concentration which would completely inhibit phospholipase D measured under the present conditions. In the rumen itself soluble proteins are usually broken down by the proteolytic activity of micro-organisms, but the susceptibility of phospholipase D to these enzymes is unknown.

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