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The Chemistry of Xanthine Oxidase

9. AN IMPROVED METHOD OF PREPARING THE BOVINE MILK ENZYME*

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A previous publication from this Institute described the preparation of crystalline xanthine oxidase from cow's milk (Avis, Bergel & Bray, 1955). The procedure gave poor overall yields and the specific activity of the product varied between wide limits. Moreover, certain stages during large-scale preparations were technically troublesome.

Because of our considerable requirements for xanthine oxidase to be used in biological (cf. Haddow, de Lamirande, Bergel, Bray & Gilbert, 1958), physical and chemical studies (see previous papers of the present series), it was most desirable to develop methods which would lead to an increased yield of enzyme and, if possible, improved specific activity and purity. The following method has consistently given very good yields of enzyme with high specific activity, provided that the buttermilk, as starting material, was fresh.

MATERIALS AND METHODS

Buttermilk. This was obtained from the National Institute for Research in Dairying, Shinfield, near Reading, Berks.

Chemicals. Where available, AnalaR-grade materials have been used throughout (British Drug Houses Ltd. or Hopkin and Williams Ltd.), exceptions being xanthine (Roche Products Ltd., Welwyn Garden City, Herts.) and Sephadex G-25 (Pharmacia, Uppsala, Sweden). All water, including that used in the preparation of the calcium phosphate (see below), was distilled and then deionized by means of an ion-exchange column (Amberlite mixed-bed resin).

Phosphate buffers were prepared by dilution of the 1 M buffer (approx. pH 6·0) described by Avis *et al.* (1955). Pyrophosphate buffer (0·1 M) was prepared by dissolving 26·8 g. of tetrasodium pyrophosphate decahydrate and 13·2 g. of disodium dihydrogen pyrophosphate in water and diluting to 1 l. Assays were carried out according to Avis *et al.* (1955), except that xanthine was stored at room temperature as a 10 mM solution in 20 mN-NaOH, this being diluted 100-fold with 50 mM-pyrophosphate buffer, pH 8·1, immediately before use.

Activity. This is the product of the rate of oxidation of xanthine ($\Delta E^{1\,cm.}_{295}$/min.; see Avis *et al.* 1955) and the dilution of the sample used in the assay.

Specific activity. This is given as the ratio, activity/$E^{1\,cm.}_{450}$, and is the 'AFR' value of Avis *et al.* (1955).

Yields. These are calculated from the total activity (activity × volume of sample) at any particular stage and are referred to the total activity present in the buttermilk.

Purity. The E_{280}/E_{450} ratio was taken as a simple indication of the purity of the samples. The lowest published value is 5·0 for the twice-recrystallized enzyme (Avis *et al.* 1955). Higher values are taken as indicating contamination of the enzyme with colourless protein.

Sedimentation analyses. These were carried out as described by Avis, Bergel, Bray, James & Shooter (1956).

* Part 8: Bray, 1961.

Calcium phosphate. Brushite (prepared by the method of Tiselius, Hjerten & Levin, 1956), obtained from 10 l. each of 50 mM-$CaCl_2$ and 50 mM-Na_2HPO_4, was mixed with 9 l. of water and to the well-stirred suspension was added, in portions, 200 g. of NaOH. The mixture was heated to 60° and this temperature maintained for 45 min. The precipitate was washed with water a number of times (batchwise with decantation) to remove fine particles and excess of alkali, i.e. until the rapidly settling precipitate left an almost clear, neutral, supernatant fluid. Alternatively, the calcium phosphate can be washed with water on a Buchner funnel until free from alkali and then suspended in water to remove the fine particles. The adsorbent is stored at about 4° under water or dilute (10–50 mM) phosphate buffer and used within 1 month. It must be emphasized that for optimum results it is essential to remove all the fine particles and to pack the adsorbent evenly. The latter can be achieved by fitting the column with an extension tube, filling the former with 0·2 M-phosphate buffer and adding the required amount of calcium phosphate in one portion. The proportion of buffer to adsorbent is not critical but the slurry should be thin enough to allow the calcium phosphate to settle as discrete particles. Dilution of the stock suspension by further 0·2 M-buffer may thus be required. A layer (1–2 cm.) of calcium phosphate should be formed before the outlet of the column is opened.

Moderate vibration of the column will aid packing: a simple method is to clamp to the same stand a stirrer motor to which has been attached an eccentrically mounted weight, e.g. a large rubber bung. The prior removal of air from the calcium phosphate by suction is also helpful. It is preferable to carry out the packing, adsorption, washing and elution without the aid of pressure as normally flow rates are only temporarily increased by this procedure and channelling is more frequent. The column should be prewashed until the pH of the effluent is the same as that of the 0·2 M buffer applied.

Purification procedure. Scheme 1 gives the outline of the method with most of the practical details, but special points are elaborated below. The essential features are proteolytic digestion followed by $(NH_4)_2SO_4$ fractionation in the presence of butan-1-ol and finally an adsorption stage with calcium phosphate. This is reminiscent of the purification procedure given by Klenow & Emberland (1955), but differs from it in a number of important respects, which include the addition of salicylate, EDTA and cysteine, combination of the butanol and $(NH_4)_2SO_4$ stages and the application of displacement chromatography.

The final step, chromatography on calcium phosphate, is the only one that requires detailed explanation but the following comments may be made on the earlier stages. (*a*) Salicylate should be added as soon as possible, e.g. directly to the milk before churning. (*b*) Other than during the digestion stage, the enzyme should be kept at 0–4°. (*c*) The precooled butanol should be added slowly, and all solids added in portions to minimize local high concentrations of the reagents. (*d*) The separation of the butanol-occluded precipitates (which have lower densities than the aqueous phase) is obviously aided by centrifuging, but the ease with which this can be done depends on the scale of the preparation and the facilities available. Thus, on a small scale, instead of leaving the treated digest (stage AM 2a) for 16 hr. one could centrifuge after about 2–3 hr. It is often convenient, however, to leave overnight and, if necessary, centrifuge only the top layer. (*e*) On a small scale, dialysis has been replaced on occasions by gel filtration through Sephadex G-25 (cf. Porath & Flodin, 1959), which speeds up the removal of $(NH_4)_2SO_4$ or the change of buffer. There appears to be no reason why this cannot be used on a large scale although this has not yet been done.

With reference to the adsorption step, the application of the crude enzyme (clarified by centrifuging if necessary) to the column in 0·2 M-phosphate buffer results in the displacement of nearly all the extraneous protein by the xanthine oxidase. One procedure is to determine the capacity of the adsorbent on a small scale by a separate experiment and then pack the column, with an excess of about 20% over that required to adsorb the amount of enzyme to be chromatographed. An alternative method (which was used here) is to apply the crude enzyme solution to the column until the calcium phosphate is almost completely saturated with xanthine oxidase, that is, until the brown colour has nearly extended to the bottom. (One must allow for the subsequent adsorption of the enzyme in the column but not yet bound.) If too much of the enzyme solution has been applied, then some will escape in the

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Buttermilk — AM 1
- 1 ml. of 20% (w/v) sodium salicylate/l.

AM 1a
- Titrated to about pH 7·5 with solid $NaHCO_3$ (17 g./l.) + cysteine hydrochloride (0·3 g./l.) and EDTA (0·37 g./l.). Digested with pancreatin (1·6 g./l.) for 3½ hr. at 37°.

Digest — AM 2
- Cooled to −2–0°. Well stirred and treated with 0·167 vol. of butan-1-ol (precooled to −20°) and 190 g. of solid $(NH_4)_2SO_4$/l. of digest.

AM 2a
- Allowed to stand 16 hr. or more at −4–4°. Clear aq. layer (approx. 60% by vol.) was run off and remainder centrifuged to obtain further aq. layer.
- ——Precipitate and butan-1-ol layer discarded.

Aqueous layer — AM 3
- Well stirred and a further 110 g. of $(NH_4)_2SO_4$/l. of aq. layer was added. Left for 1–2 hr. at −4–4°; clear aq. layer run off, top layer centrifuged and ppt. collected.
- ——Aq. layer discarded.

Precipitate — AM 4
- Suspended in and dialysed against 0·2 M-phosphate buffer, pH 6, containing 0·2 mg. of sodium salicylate/ml. Alternatively, $(NH_4)_2SO_4$ removed by gel filtration (see text).

Non-diffusible fraction — AM 4a
- Adsorbed on a column of calcium phosphate (see text). Washed with 0·2 M-phosphate, pH 6. Eluted with 0·1 M-pyrophosphate, pH 7, containing 0·2 mg. of sodium salicylate/ml. Temperature 4° throughout.

Final product — AM 5

Scheme 1. Xanthine oxidase preparation.

effluent, but this may be collected and purified by repeating the procedure. A column 10 cm. × 6·4 cm. diam. should adsorb 0·75–1·0 g. of enzyme. Two to four bed volumes of the 0·2 M buffer are sufficient for washing.

Occasionally, during the elution stage, small quantities of enzyme are not removed from the adsorbent by the pyrophosphate buffer. This appears to be due to changes in the calcium phosphate, possibly as the result of prolonged storage. These residual quantities of xanthine oxidase can be eluted with 1 M-phosphate buffer: their purity may be quite high but this buffer will also elute a green protein (probably lactoperoxidase), which is sometimes present.

DISCUSSION

Yield of enzyme

Since the findings of Bergel & Bray (1956, 1959) that salicylate stabilizes the enzyme, this compound has always been added to the buttermilk immediately it has been prepared and at subsequent stages where necessary. The use of salicylate and EDTA may have some favourable effect on the overall yield, but the very high yields depend almost entirely on the addition of cysteine before the digestion stage. The rationale for this procedure is that cysteine and β-mercaptoethanol (at about 2 mM) release the xanthine oxidase from its association with particulate matter (see Baillie & Morton, 1958). In our experience the bound form of the enzyme generally comprises about 40% of the total activity of the buttermilk and this value appears to be correlated with that fraction of enzyme which is invariably lost during decaseinization (cf. Avis *et al.* 1955; Klenow & Emberland, 1955; Horecker & Heppel, 1949). The almost complete recovery of activity up to stage AM 4a represents a near quantitative yield and is not due to activation phenomena. It should be borne in mind that xanthine oxidase released from the particles may differ in some respects from the enzyme already present in solution although, as yet, there is no evidence that this is so.

Losses incurred during the precipitation stages are due almost entirely to the difficulty of separating a small volume of low-density precipitate (with occluded butanol) from a large volume of liquid. Such difficulties vary with the scale on which the preparation is carried out, being least marked when starting with about 1–2 l. of buttermilk. The ability of the chromatographic stage to deal adequately with large quantities of extraneous protein allows the complete recovery of enzyme to be effected by the use of wide limits of saturation with ammonium sulphate.

Low flow rates and channelling of the calcium phosphate column present the greatest troubles, the difficulties usually increasing as the scale is increased. Careful attention to the packing of the columns, in the manner indicated above, and to the clarification of the crude enzyme solution (AM 4a), should minimize troubles of this kind.

The enzyme is usually stored at stage AM 4a and chromatographed as required; for the large-scale preparations it is therefore possible only to estimate the average overall yield as being between 70 and 80%. In small-scale preparative experiments, where all the enzyme has been chromatographed immediately, the yield has been as high as 90%. In terms of weight, such yields can represent as much as 8 g. of enzyme from 20 l. of buttermilk.

Specific activity

This is not entirely independent of the yield since the latter is based on activity measurements, at least for the earlier steps. We have consistently obtained final products with activity/$E^{1\,cm.}_{450}$ ratios of 100, and occasionally the values have been as high as 115–120. The specific activity of the final product, and to some extent the yield, depends much more on the prior treatment of the buttermilk rather than on details of the procedures given above. Thus where there has been a delay of some hours in the delivery of the buttermilk (with consequent storage at or above room temperature) both the specific activity and yield of the product have been decidedly lower. Although one or two batches of enzyme prepared by the present method

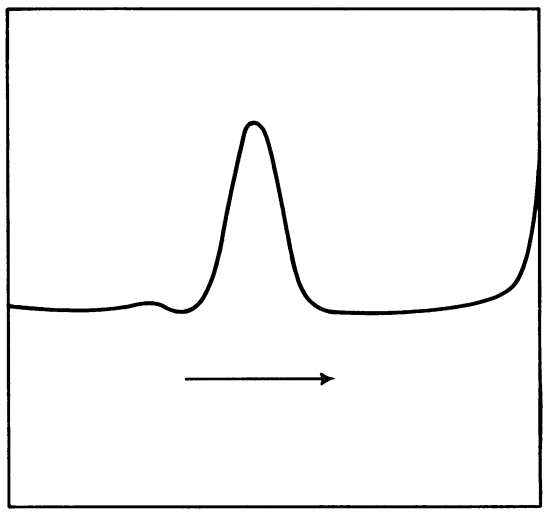

Fig. 1. Sedimentation of xanthine oxidase. Conditions: final product (stage AM 5); sedimentation in 0·1 M-pyrophosphate buffer, pH 7·0. Drawn from a photograph taken after 103 min. at 42000 rev./min. Sedimentation coefficient of the main peak is 10·2 s. The smaller peak represents 4% of the total solute. There is also a very small amount of a component sedimenting faster than the xanthine oxidase. The arrow shows the direction of sedimentation.

proved exceptionally unstable (cf. Bray, 1959, for a possible explanation), in all other respects the xanthine oxidase appears identical with that produced by the method of Avis *et al.* (1955).

Purity of the final product

On the basis of both the E_{280}/E_{450} ratio and ultracentrifugal analyses (Fig. 1) the purity of the final product normally lies in the range 85–95%. The chromatographic stage leads to a decrease in the E_{280}/E_{450} ratio from about 15–20 to 5·4–6·0. The method as given above represents a compromise between yield and purity: it is possible, of course, for one to be improved at the expense of the other. The lower purity value quoted may be considered adequate for some purposes, e.g. if the enzyme is to be stored for a considerable period and high stability is required. Where 'ultra-pure' enzyme is required and some 'losses' can be accepted, either the adsorption may be prolonged until only xanthine oxidase is retained on the column (virtually all other proteins being entirely displaced) or, by the use of a fraction collector, only that part of the eluate having a low and constant E_{280}/E_{450} ratio need be retained. Alternatively, the purity may be increased by a second ammonium sulphate fractionation within narrower limits than those given in Scheme 1.

SUMMARY

1. A relatively simple preparation of bovine milk xanthine oxidase is presented.

2. Yields of 70–90% have been made possible by the use of cysteine to release the enzyme from a particulate complex.

3. The average specific activity (activity/$E^{1\,cm.}_{450}$ ratio) of the product is 100.

4. The purity is 85–95% and indications are given how this may be improved at the expense of yield.

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The Effect of Short-Chain Fatty Acids on Blood Glucose Concentration in Sheep

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The energy requirements of ruminants are supplied largely by short-chain fatty acids produced by fermentation of the food in the reticulo-rumen sac and other parts of the alimentary tract. Owing to the nature of the food usually consumed and to these fermentations it is improbable that much sugar is normally absorbed by these animals; the only likely source is polysaccharide synthesized by bacteria and protozoa. The available evidence suggests that their carbohydrate requirements are met largely by gluconeogenesis. It can be predicted, from established metabolic reactions, that propionate is convertible into carbohydrate in the

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