Evidence for the Presence of Several Lipases in Cow's Milk

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Skim milks containing sodium chloride (0.75M) were centrifuged at 80000g for 2 hr. and portions of the supernatants were submitted to gel filtration on columns of Sephadex G-200. Enzymes in the effluent fractions were assayed titrimetrically for their hydrolytic activities towards tributyrin, triolein and milk-fat emulsions, and triacetin solution. Summation of the measurements gave ratios of activities towards the various substrates similar to those of the original skim milks. Although only partial separation was obtained, five enzymes appeared to be present. They showed some differences in substrate specificity, but all appeared to be lipases in that they hydrolysed the emulsified substrates more rapidly than the dissolved triacetin.

Experiments with various substrates and enzyme inhibitors indicated the presence of mixtures of lipases or other esterases in cow's milk (Frankel & Tarassuk, 1956a,b; Forster, Bendixen & Montgomery, 1959; Forster, Montgomery & Montour, 1961), but the individual enzymes in such mixtures are difficult to characterize in the presence of one another because of their overlapping substrate specificities. We used gel filtration to fractionate the tributyrinase activity of cow's milk and to aid the identification of different enzymes (Downey & Andrews, 1965a, 1966). Tributyrinase activity in cow's milk is associated mainly with the casein micelles, but addition of sodium chloride to the milk dissociated the enzyme-micelle complexes and gave smaller, soluble, complexes. These appeared to have mol.wt. about 350 000, and still contained tributyrinase activity. They dissociated during gel filtration under certain conditions and the tributyrinase elution patterns then indicated the presence of three partially separated enzymes that together contributed most of the activity.

Further gel-filtration experiments combined with substrate-specificity studies now indicate the presence in cow's milk of a more complex mixture of lipases than we hitherto supposed. A brief report of these results has been published (Downey & Andrews, 1965b).

MATERIALS AND METHODS

Enzyme preparations from cow's milk. A Lister cream separator was used to prepare skim milks from uncooled raw milks about 1 hr. after their collection from individual

Friesian cows. Enzyme preparations were the supernatant fractions obtained from the skim milks after the addition of NaCl (final concn. 0.75M) and centrifugation in a Spinco model L ultracentrifuge (rotor 30) at 80000g for 2 hr. at 2°. The supernatant was collected through a hole pierced in the Lusteroid centrifuge tube about 1 cm. above the casein pellet, and was used for gel filtration within 2 hr. of its preparation. The E_{340} of the preparations was taken as a measure of their casein-micelle content, and any with E_{340} greater than 0.2 were discarded. The solutions contained 0.12–0.14 mg. of casein N/ml. (determined by the method of Rowland, 1938), equivalent to 3–3.5% of the casein N originally present in the skim milks.

Enzyme assays. Triacetin, tributyrin, triolein and gum acacia were obtained from British Drug Houses Ltd., Poole, Dorset. Milk fat was the top layer obtained by centrifuging fresh milk at 20000g for 30 min., and was stored at –20° in a stoppered tube. Emulsions were prepared from substrate (tributyrin, 10 ml.; triolein, 1 ml.; or milk fat, 0.5 g.), gum acacia (10 g.) and ice-cold water (80 ml.) as described by Downey & Andrews (1965a). The pH of the resultant emulsions was adjusted to 8.5 with M-NaOH, and water was added to give final volumes of 100 ml. The emulsions were used on the day they were prepared. For triacetin solution, the triglyceride (1 ml.) was dissolved in water (100 ml.) and the pH adjusted to 8.5.

Enzyme activity was measured by continuous titration with MM-NaOH of the acid liberated from the appropriate substrate, by using a Radiometer Titrator type TTT1c, coupled to a Radiometer Titrigraph type SBR2c (Radiometer, Copenhagen, Denmark). The assay mixture was contained in a water-jacketed cell maintained at 25° and mechanically stirred and bubbled with CO₂-free N₂. A description of the assay method is given by Downey & Andrews (1965a, 1966), and includes details of the precautions we found necessary to eliminate electrostatic effects when using MM-NaOH as titrant.

Assays were performed either with substrate emulsion (2 ml.) or with triacetin solution (1 ml.) in a total volume of 5 ml. at pH 8.5. Rates of acid production were linear with time, proportional to the amounts of enzyme added

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and unchanged when the amounts of substrate were doubled. Measurements were reproducible to within $\pm 5\%$. One unit of activity is taken as the amount of enzyme liberating $1\mu\text{equiv.}$ of acid from the substrate/min., and with 1mM-NaOH as titrant the method was used to measure 10–70 milliunits of enzyme activity.

Gel filtration. Columns ($50\text{cm.} \times 2.5\text{cm. diam.}$) of Sephadex G-200 [Pharmacia (Great Britain) Ltd., London W.13] were packed in the cold with the swollen gel, and equilibrated with 0.75M-NaCl containing 25mM-MgCl_2 . Effluent was collected in fractions (5 ml.) by using a collector fitted with a siphon. All columns were run at $2-5^\circ$ and flow rates were 10–15 ml./hr.

RESULTS

Enzyme preparations from the milks of four cows had tributyrinase activities in the range 0.55–0.87 unit/ml., these being about 65% of the activities of

the original skim milks. The activities of the preparations decreased by 15–20% during 1 day at 2° , whereas the skim milks lost only 5% under similar conditions. The skim milks and the enzyme preparations obtained from them showed similar variations in their relative activities towards different substrates, which were in the following proportions: tributyrin, 100; milk fat, 80–100; triolein, 40–50; triacetin, 8–12. No significant differences were observed when an enzyme preparation was assayed with separately prepared emulsions of any of the first three substrates, or with different solutions of triacetin.

Portions (10–30 ml.) of enzyme preparations from three milks were submitted to gel filtration and samples of each effluent fraction were assayed, one with tributyrin emulsion and another with milk-fat emulsion, triolein emulsion or triacetin solution as

Fig. 1. Gel-filtration and substrate-specificity studies on enzyme preparations from one skim milk. The elution diagrams show (a) protein (—, E_{280}) and tributyrinase activity (----) in a 10 ml. sample; (b) detail from (a) with the activity towards tributyrin emulsion (●) compared with that towards milk-fat emulsion; (c) activities towards tributyrin (●) and triolein (Δ) emulsions in a 20 ml. sample; (d) activities towards tributyrin emulsion (●) and triacetin solution (○) in a 30 ml. sample. Activities towards tributyrin and milk fat were assayed with 1–2 ml. portions of each fraction, and activities towards triolein and triacetin with 3 ml. or 3.5 ml. portions. Suggested positions of different enzymes are indicated by numbered arrows.

Fig. 2. Enzyme activities from Fig. 1 expressed as percentages of the corresponding tributyrinase activities and plotted with the same abscissae. The numbered arrows, in the same positions as in Fig. 1, show suggested positions of different enzymes. Activity towards (a) milk fat, (b) triolein and (c) triacetin is shown.

substrate. The assays on each fraction were performed in rapid sequence to minimize possible effects of enzyme instability, and each column run and the assay of fractions from it was completed in 1 day. Assay of each run with more than two substrates in 1 day was impracticable because of the time involved. Recoveries of enzyme activity ranged from about 40% for 10ml. samples to 70% for 30ml. samples.

Elution diagrams for preparations from one milk (Fig. 1) illustrate the results obtained. Enzyme activity was detected in a similar region of overlapping peaks in each case, but preparations from different milks showed small differences in the relative heights of the peaks. Summation of the activities towards tributyrin, milk fat, triolein and triacetin shown in Fig. 1, and correction for the different amounts of enzyme preparation used, give the proportions 100:100:40:9 respectively, similar to those found for the original enzyme preparation.

Activity ratios relative to tributyrinase activity in the effluent fractions of Fig. 1 are shown in Fig. 2.

DISCUSSION

The gel-filtration behaviour of tributyrinases in crude enzyme preparations from cow's milk depends very much on experimental conditions, because of concentration-dependent interactions between the enzymes and other proteins (Downey & Andrews, 1965*a*, 1966). The presence of at least three tributyrinases in the preparations used in our earlier experiments was only revealed when small samples were used for gel filtration, and dilution during chromatography was sufficient to minimize the interactions. Dissociation of the soluble enzyme complexes in the crude enzyme preparations occurred more readily when the casein-micelle content of the preparations was decreased by further centrifugation. The present experiments show that, when the sample size of such preparations relative to the column size in gel filtration was increased considerably beyond the optimum for the earlier experiments, useful separations of the constituent tributyrinases were still obtained. Recoveries of enzyme activity in the column effluents were better with the larger samples (up to 50% from the original skim milks) and the individual column fractions generally contained sufficient enzyme for assays with two substrates.

The ratios of hydrolytic activities towards the different substrates obtained by summation of measurements on the effluent fractions agreed well with the ratios for the corresponding skim milks. Presumably, therefore, the mixture of enzymes revealed by gel filtration is similar to the mixture in skim milk. However, the results give only semi-quantitative information about the relative amounts of the various enzymes and their substrate specificities because separation was incomplete and assay conditions with the various substrates were not necessarily optimum.

All the enzymes apparently have the characteristic property of lipases of greater activity in hydrolysis of emulsified triglycerides than in hydrolysis of dissolved triacetin (Figs. 1 and 2). However, the enzymes designated 1 and 5 were clearly distinguished from the remainder by differences in substrate specificity, and enzyme 1 differed from enzyme 5 in its markedly greater activity towards milk fat than towards tributyrin. In preparations from all three milks examined, a major part of the lipase activity was evidently due to enzymes that were poorly separated from one another and hardly distinguishable by their substrate specificities. There were at least two of these enzymes, including enzyme 2, and possibly more. For example, most of the elution diagrams were

similar to those of Fig. 1 in suggesting the presence of the two enzymes 3 and 4. Variations in the spacing between these enzymes and the remainder, as suggested by Fig. 1, may have been due to delayed dissociation of soluble enzyme complexes when the larger samples were used.

With smaller samples, as in Fig. 1(a) for example, enzymes 2, 3 and 4 were eluted in similar positions relative to the protein diagram, as were the three tributyrinases with estimated mol.wt. 112000, 75000 and 62000 respectively described by Downey & Andrews (1965a). Of the newly recognized enzymes, enzyme 1 was eluted slightly later than the milk globulins and enzyme 5 nearly coincided with β -lactoglobulin, these results indicating mol.wt. for them of 150000–180000 and 35000–40000 respectively. Results of the present experiments contrast with those of the earlier ones in that tributyrinase was not detected in the column effluents at the void volume, but the sedimentation of more protein from the enzyme preparations may account for this. A strong buffering effect resulting from the use of large samples precluded enzyme assays on the effluent fractions containing low-molecular-weight material (Downey & Andrews, 1965a).

The isolation of a homogeneous lipase preparation from cow's milk by Fox & Tarassuk (1968) seems at first sight to be evidence against the heterogeneity of milk lipase. However, this preparation and the soluble enzyme complexes in our crude preparations (Downey & Andrews, 1966) seem to be markedly alike at least in size. At low

ionic strength the homogeneous preparation was eluted from Sephadex G-200 before human γ -globulins (Fox & Tarassuk, 1968), and in terms of a calibration based on 'typical' globular proteins (Andrews, 1965) this suggests a mol.wt. about 300000. Our estimate for the molecular weight of the soluble complexes, based on their gel-filtration behaviour, was about 350000. Whether or not the homogeneous preparation resembles the soluble complexes in its dissociation at high ionic strengths remains to be determined.

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