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Journal of Molecular Catalysis B: Enzymatic 4 (1998) 161–166

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Clarification of the reaction sequence of lamb pregastric lipase catalyzed hydrolysis of dibutyryl-1,2-propanediol

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Received 25 August 1997; revised 25 September 1997; accepted 26 September 1997

Abstract

Lamb pregastric lipase, extracted from the tongue root and epiglottis of suckling lamb, has been used to catalyze the hydrolysis of dibutyryl-1,2-propanediol. Reactions were carried out at 35°C and initial pH ~ 7.0. Varying rates of reaction were produced by increasing the amount of enzyme added relative to the amount of diester. A system rate parameter, $\Delta pH/\Delta t$, the change in pH (ΔpH) from time zero to the time when the sample was removed (Δt), has been applied to the experimental data. The monoesters, 1-butyryl-propan-2-ol and 2-butyryl-propan-1-ol have been identified by ^{13}C NMR as products of the reaction. The 1-monoester is formed by stereoselective enzymic hydrolysis of the diester. The 2-monoester is formed by non-catalyzed acyl migration with a rate constant equal to 9.72×10^{-5} s^{-1}. The equilibrium composition is 33.2% for 1-monoester and 66.8% for 2-monoester. © 1998 Elsevier Science B.V.

Keywords: Acyl transfer; Diacyl-1,2-propanediol; Enzyme catalysis; Hydrolysis; Acyl-1(2)-propanol; Lamb; Pregastric lipase

1. Introduction

Enzyme products prepared in New Zealand, extracted from the tongues of lambs and calves, have been commercially available to the food industry for some years. The preparations, from N.Z. Rennet, have found particular application in the modification of the textural and flavor properties of foodstuffs, including cheeses and baking mixtures.

Pregastric enzymes, being mammalian enzymes, are generally regarded as safe if they are extracted from disease-free animals. Moreover, if they are used as supplied by the manufacturer, i.e. without further purification, they meet the requirement of commercial viability. Therefore, a knowledge of their physico–chemical properties should extend their potential for use in rearranging the fatty acids in triglycerides and esters to produce new triglycerides or esters and also to add or remove fatty acids to change their composition.

Our early work on the kinetic characterization of pregastric lipase-mediated hydrolysis of the short-chain lipid tributyrylglycerol (TBG) [1] and of other monoacid triglycerides [2] subsequently led to analysis of reaction products. During the course of the hydrolysis of TBG we initially observed high ratios of 1,3-diacylglycerol to 1,2-diacylglycerol [3], which led to consideration of the possibility of an enzyme

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PII S1381-1177(97)00033-7

with *sn*-2 selectivity. However, such enzymes are extremely rare [4]. Closer examination of reaction progress revealed an unusual pattern of reaction products. Early in the reaction the *rac*-1,2-isomer predominated, but later the 1,3 isomer became the predominant diacylglycerol in the reaction mix. A mechanism was proposed in which the 1,2-isomer was produced by a primary hydrolysis pathway, and this was followed by a non-enzymatic intramolecular acyl transfer of the *sn*-2 carboxylic acid to the free exterior position of the *rac*-1,2 diacylglycerol.

In this investigation we have used a commercial source of lamb pregastric lipase (LPGL) to catalyze the hydrolysis of dibutyryl-1,2-propanediol (**1**). We have shown [5] that the relative values of V_{max} for hydrolysis of TBG: dibutyryl-1,2-propanediol: dibutyryl-2,3-propanediol are 100:35.4:12.5 and the values of K_m are in the range 0.24–0.39 mM. For each substrate, mono-hydrolysis stoichiometry was observed. The reactions were carried out at pH 6.5, 35°C and used < 1 mg of enzyme in a reaction volume of ~ 40 ml.

The diester **1** presents a rather lipid-like structure to the enzyme with the ester linkages (and chains) lying close together. In keeping with its greater hydrophobic character compared with the 1,3-diester (arising from increased steric exposure of the methyl group) it is more easily hydrolyzed [5]. It was therefore believed to be a satisfactory model for dibutyryl-1,2-diacylglycerol, since it mimics the spatial association of the pairs of acyl chains, but in the absence of a free position it was thought that acyl transfer might be restricted. However, such is not found to be the case. The results provide strong evidence for the potential versatility of LPGL in biotechnological processing.

2. Experimental

2.1. Materials

Freeze dried lamb pregastric lipase (LPGL) was supplied by New Zealand Rennet. The preparation had been filter-sterilized and freeze dried and was supplied without the lactose extender of the standard commercial product. It was used as supplied and had a lipase activity of 5.5 μmol free fatty acid min^{-1} mg^{-1} (40°C, pH 6.5, 8.5 mM TBG). TBG and bis[tris(hydroxymethyl)methylamino]propane, Bis–Tris–propane, were from Sigma.

Dibutyryl-1,2-propanediol was prepared by acylation of 1,2-propanediol (BDH LR reagent). A 10% excess of butyryl chloride was added to the diol (dried and pre-distilled) in a flask, cooled in ice and fitted to a vacuum distillation apparatus. During stirring the pressure was frequently reduced and as the rate of gassing decreased and the two liquid phases merged, the temperature was allowed to increase. After 2 h, low pressure distillation was carried out. The purity of the product ($> 98.5\%$) was confirmed by ^{13}C NMR. The product was a low viscosity colorless liquid at room temperature and was stored in a sealed flask at 4°C.

2.2. ^{13}C NMR analysis and quantitation

The ^{13}C NMR spectra were obtained by using $CDCl_3$ solutions and a carefully shimmed Bruker AC200 spectrometer. A Bruker AM400 instrument was also used occasionally for confirmation of integration. Assignment of the ^{13}C and ^{1}H NMR spectra were aided by DEPT 135 and XHCORR. Species were identified by the patterns of their shifts and their intensities. Assessment of mole percentage composition of each species was typically by peak height summation of appropriate sets of oxygenated carbon resonances in the 64–72 ppm shift range. Quantitation was usually replicable to better than $\sim \pm 2\%$ absolute.

2.3. Product analysis of reaction sequence

Example 1 employed diester **1** (33.2 mM, 430 mg) emulsified in 60 ml of 200 mM Bis–Tris–propane at 35°C, initial pH (pH_i) = 6.98,

to which ~152 mg LPGL were added. The change in pH was monitored over the next 140 min. Samples of the reaction mixture, 20 ml, 20 ml and ~18 ml, were removed at 33, 73 and 135 min, respectively. Care was taken with the last sample to avoid disturbing material which had adhered to the vessel walls. Each sample was immediately extracted with cool $CHCl_3$, and processed for ^{13}C NMR examination.

Reaction conditions for example 2 were similar to those for example 1 with the following changes: 34.7 mM **1** (450 mg), $pH_i = 6.97$, 190 mg LPGL. The pH was monitored for 84 min. Samples were removed at 11, 26.5 and 84 min and analyzed by ^{13}C NMR quantitation.

The conditions for example 3 were designed to produce very fast reaction rates. The reaction mixture employed 430 mg **1** (49.7 mM) in 40 ml Bis–Tris–propane buffer at 35°C, $pH_i = 6.96$, and 2.6 g LPGL. The pH was monitored over a period of 9 min, at which reaction time a single 30 ml sample was extracted from the center of the reaction vessel, away from the gel which coated the sides. The $CHCl_3$ extract from this sample was first analyzed by optical rotatory dispersion and then by ^{13}C NMR quantitation to determine percentage composition.

3. Results and discussion

3.1. Product analysis of reaction sequences

The profile of pH versus time for example 1, and the data for molepercent composition of samples removed during the course of the reaction, are plotted in Fig. 1. It is seen that the pH decreases from 6.98 to 6.65 during the 135 min reaction period, but at ~70 min the rate of decrease in pH slowed suddenly and the substrate was almost exhausted. Following this, while the proportion 1-butyryl-propan-2-ol (**2**) started to decrease, that of 2-butyryl-propan-1-ol (**3**) continued to increase slowly.

In the hydrolysis of TBG, the occurrence of different rate constants for the appearance of

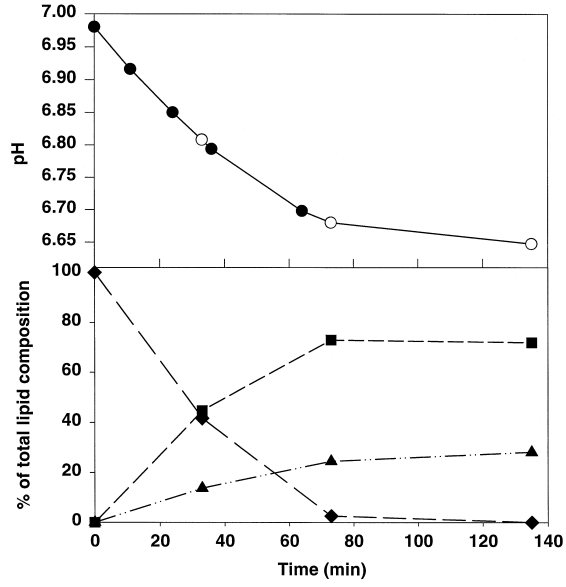
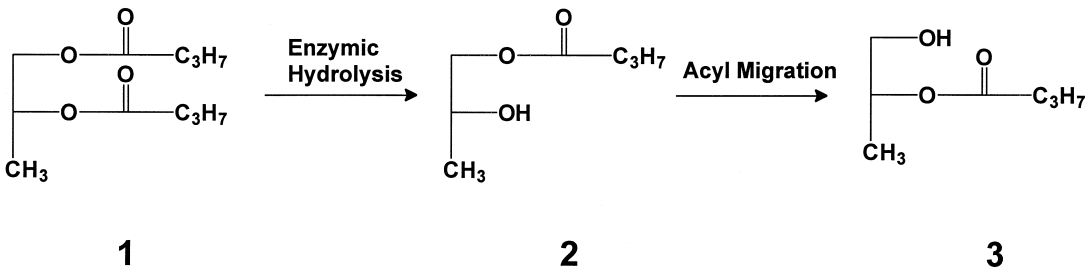


Fig. 1. LPGL catalyzed hydrolysis of 33.2 mM dibutyryl-1,2-propanediol, 35°C, 200 mM Bis–Tris–propane, 152 mg LPGL. Upper plot: pH versus time data. Reaction mixture sample points are shown hollow. Lower plot: ^{13}C NMR product analysis with time; 1,2-diester (◆), 1-monoester (■), 2-monoester (▲).

two separate products, 1,2-dibutyrylglycerol and 1,3-dibutyrylglycerol, was indicated by changing ratios of these isomers as the level of catalytic activity was varied [3,6]. This observation led to the suggestion of co-participation of both specifically catalyzed and non-catalyzed pathways. By analogy, it seems that the 1-monoester **2** arises from LPGL catalyzed hydrolysis of the 1,2-diester, whereas the 2-monoester **3** is formed primarily by acyl migration from the 1-mono isomer (Scheme 1). If this were so, then a faster reaction sampled at shorter times should show a higher proportion of **2**.

The experimental conditions described for example 2 (Fig. 2) meet these requirements and show that the drop in pH is the same as that seen for example 1, but the time for this reaction is now reduced to 84 min.

Although the reaction profiles for the presence of the **2** and **3** appear very similar to those of the previous example, the ratio of **2**:**3** (which was a very sensitive measure of shifts in relative quantity) is slightly higher (thus, 3.7 cf. 3.0 at



Scheme 1. Reaction sequence of LPGL catalyzed hydrolysis of 1,2–dibutyrylglycerol.

74 and 73 min, respectively). It is also seen that the isomer ratio is progressively higher in 1-mono isomer **2** for shorter reaction times. Although there is a lag in the appearance of the 2-monoester **3** (which depends on the product of the primary hydrolysis for its substrate), once hydrolysis has commenced, then production of **3** by isomerization approximately matches the continued rate of production of **2**. In order to resolve these two rates more clearly, a very fast reaction was required such that the production of 1-monoester by catalyzed hydrolysis was much faster than the acyl transfer rate which produces **3**.

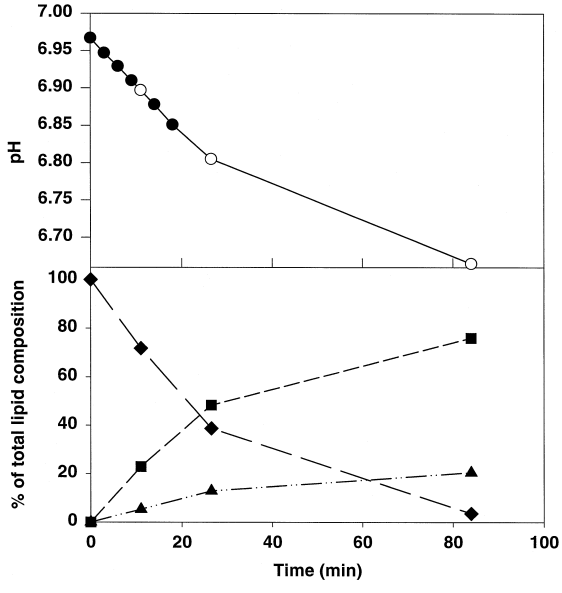


Fig. 2. LPGL catalyzed hydrolysis of 34.7 mM dibutyryl-1,2-propanediol, 35°C, 200 mM Bis–Tris–propane, 190 mg LPGL. Upper plot: pH versus time data. Reaction mixture sample points are shown hollow. Lower plot: ^{13}C NMR product analysis with time; 1,2-diester (♦), 1-monoester (■), 2-monoester (▲).

We had previously found [5] that the relative rates of hydrolysis of TBG:**1** were ~2.6:1 under the conditions of the experiments in this investigation. In the fastest reaction studied for hydrolysis of TBG and subsequent determination of reaction products [6] we had used 1080 mg of LPGL. In order to gain a similar rate for hydrolysis of the diester we employed 2600 mg LPGL. This amount of enzyme was the largest amount employed in any single experiment, and aggregation and gel formation in the reaction mixture were extremely pronounced.

The pH profile for example 3 (not shown) was similar to those given for examples 1 and 2, except that the reaction was approaching completion at the time of sampling (9 min). The $CHCl_3$ extract of this sample yielded 297 mg of light oily material, whose composition, as determined by ^{13}C NMR quantitation was 0% **1**, 85.9% **2** and 14.1% **3**.

Although the substrate diester in the sample volume was exhausted, the smaller decrease in pH (6.96–6.64 over 9 min) suggests that some unreacted substrate was sequestered in the gel. Under these complex physical conditions, the water-soluble enzyme is more likely to be active in the aqueous regions where the convective exchange required for continued operation of the hydrolysis reactions is maintained. Since the sample was taken from this well stirred and more fluid region of the reaction mixture, the likelihood of complete hydrolysis was enhanced.

3.2. The parameter $\Delta pH / \Delta t$

Amongst the trends emerging from consideration of the above examples is the apparent predominance of the 1-monoester **2** as the primary product of the LPGL catalyzed hydrolysis of **1**, together with an increase in the proportion of 2-monoester **3** as the reaction proceeded. Analysis of the ratios of these isomers as a function of reaction rate and reaction time is desirable as an indicator of the effect of reaction rate on the speciation of the monoesters produced as the primary products of LPGL-catalyzed hydrolysis. In order to outline this relationship, a method of representation of the reaction rate was required. In view of the parallel reactions possibly occurring, a simple generalized reaction rate parameter, $\Delta\mathrm{pH}/\Delta t$, was chosen. This is defined as the change in pH $(\Delta\mathrm{pH})$ from $\mathrm{pH_i}$ to the pH at the time of the sample being removed, divided by the time elapsed (Δt) before removal of that sample. Since all the hydrolysis reactions produce protons, the ΔpH in a buffer of known volume, concentration and $\mathrm{p}K_\mathrm{a}$ is a measure of the nett amount of hydrolysis that has occurred.

It should be noted that the value of $\Delta\mathrm{pH}/\Delta t$ is a crude estimate of the reaction rate, in that it is derived from many differing spans of time and of ratios of enzyme to substrate, and is always referred to the commencement of the experiment when only **1** is present, and not to the composition of the previous sample. The early contribution to the value of this parameter is largely the result of the hydrolysis of **1** to **2**, and for a known $\mathrm{pH_i}$ value, the reaction rate $(\Delta\mathrm{pH}/\Delta t)$ may be precisely calculated from the relevant pH value at time of sampling. The rate parameter is also likely to be affected by a number of physical and chemical conditions, including the rate of stirring, initial pH and temperature. Bis–Tris–propane was chosen as buffer because its $\mathrm{p}K_\mathrm{a} \sim 6.8$ (25°C) lay within the range of pH values (7.0–6.6) generally encountered during the course of the reactions. Within this pH range the linearity of buffering was maximized and the effect of the change in pH on enzymic activity [1] is minimized. Moreover, a neutral pH is likely to avoid general acid- and base-catalyzed hydrolysis of the ester substrates and to minimize their autolysis.

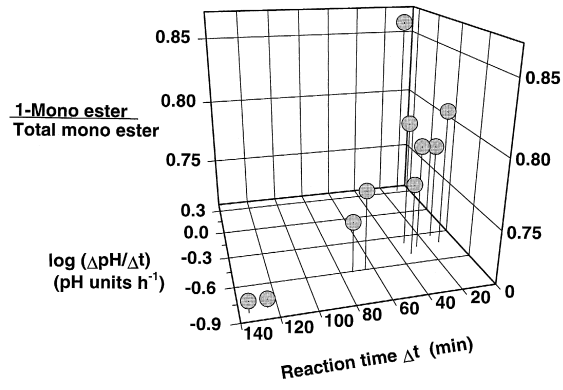


Fig. 3. Monobutyryl-1,2-propanediol isomer composition as a function of the logarithm of the average rate of change of pH $(\Delta\mathrm{pH}/\Delta t)$ and reaction time (Δt) for LPGL catalyzed hydrolysis of differing masses of dibutyryl-1,2-propanediol, $\mathrm{pH_i} \sim 7.0$, 35°C.

The evidence shows that in the catalyzed reactions, the proportion of 1-monoester to total monoesters is reduced for longer reaction times and for slower reaction rates.

A very high reaction-velocity sample provided confirmation of the preferential position for catalyzed hydrolysis, and the reaction leading to **2** was well-resolved from the acyl transfer reaction to **3**, which also occurred. We have independent evidence (not shown) for these uncatalyzed acyl migration reactions.

Fig. 3 shows the monoester molefraction composition as a function of the $\Delta\mathrm{pH}/\Delta t$ parameter, and the reaction time Δt, for a number of reactions of the diester, including examples 1–3.

3.3. Position of LPGL catalyzed hydrolysis of dibutyryl-1,2-propanediol

Fig. 3 shows a clear correlation of composition with the reaction velocity parameter and elapsed time. The data suggest a sharply selective reaction producing the 1-mono isomer, and the possibility exists that the catalyzed hydroly-

sis was exclusively selective for the 2-position. If this were so, the observed quantities of the 2-monoester suggest the rate of the acyl transfer reaction is approaching the rate of the slower hydrolyses.

Of the two ester linkages present, the 2-position is likely to be the more hydrophobic, being adjacent to a methyl, and it is possible that this position appears as a lipid-like mimic to LPGL. Just as the terminal spine-position is apparently exclusively favored in the catalyzed hydrolysis of TBG [6], so too the LPGL-catalyzed hydrolysis of **1** has a strongly preferred site.

In the case of LPGL-catalyzed hydrolysis of TBG, the mono-hydrolysis initially occurs for a substrate with two (1- and 3-) equivalent positions. Furthermore, only one of these two positions is subject to hydrolysis. The 1,2-diester product also is only singly hydrolyzed in the presence of LPGL, and only one positional product is primarily produced.

In contrast to the triacylglycerol, mono-stoichiometry for diester hydrolysis mitigates against stereoselectivity. Since all propanediol ester species (including the 1,2-diester) are individually chiral, the 1,2(2,3)-diester is present at a molecular level as approximately 50:50 (*S*)-1,2- and (*S*)-2,3-diester. There can be no strongly stereoselective hydrolysis reaction, since the stoichiometry would then be for half-hydrolysis (only half of the substrate would be available for catalysis) and only one fatty acid could be released per hydrolysis. The observed mono-stoichiometry cannot result from stereoselective hydrolysis of position 2 in the (*S*)-1,2-diester and position 3 in the (*S*)-2,3-diester, since only the one positional reaction product is observed. The sample removed from example 3 was found to be optically inactive, in agreement with expectations.

The evidence is therefore supportive of the reaction sequence shown in Scheme 1 in which LPGL exerts positional selectivity for the 1,2-diester at the 2-position and then the 2-monoester is produced by acyl transfer. In confirmation of this sequence, we have determined that the acyl transfer reaction is observed without enzymic catalysis. In 200 mM Bis–Tris–propane, pH 7.0, 35°C the rate constant for the acyl transfer reaction was 9.72×10^{-5} s^{-1} ($t_2 = \sim 2$ h) and the equilibrium composition was 33.2% **2** and 66.8% **3**. It is now seen that the mixture extracted after 135 min reaction time in example 1 (71.9% **2**, 28.1% **3**) is part-way towards this composition as it moves towards equilibrium.

Acknowledgements

We acknowledge financial assistance from Auckland University Research Committee, New Zealand Lottery Science and the New Zealand Agricultural and Marketing Research Development Trust. We are indebted to Dr. Keith Turner and the New Zealand Rennet Co. Ltd. for the kind donation of enzyme and continued interest.

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