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Journal of Molecular Catalysis B: Enzymatic 1 (1996) 143–149

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Lamb pregastric enzyme-catalysed hydrolysis of 4-nitrophenylalkanoates and monoacid triglycerides

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Received 7 September 1995; accepted 8 December 1995

Abstract

Three lamb pregastric enzymes, isolated from the commercial extract from the tongue and epiglottal region of lamb, have been used to catalyze the hydrolysis of a series of 4-nitrophenylalkanoate esters (C2–C12) at 37°C, pH 7.2 and maximum activity was obtained against the decanoate ester in all cases. Burst kinetics were observed for activity of the principal lipase component against the decanoate ester. This enzyme was also used as a catalyst for the hydrolysis of monoacid triglycerides (C4:0 to C10:0) at 35°C, pH 6.5 and maximum activity was obtained against tributyrin (C4:0). A suggestion is made for orientation of ester substrates within the active site of the enzymes.

Keywords: Lamb pregastric enzymes; 4-Nitrophenylalkanoates; Monoacid triglycerides; Hydrolysis

1. Introduction

Although pregastric enzymes of ruminant origin have been used for many years as flavor enhancers of cheeses, very little is known about their activity against individual substrates. We have discovered that the lamb pregastric enzyme preparation available from the New Zealand Rennet Co. Ltd, Eltham, New Zealand, contains three active enzymes. Neither the separation nor purification of these components has been optimised, but the fractions used in the studies which follow were homogeneous and contained ≈ 100% protein by weight. The account which follows focuses upon the determination of preferred substrate-size for hydrolysis reactions of esters and lipids catalysed by these fractions. As a result, seeming differences in carbon chain length of preferred ester substrates have been rationalized.

Studies designed to elucidate the physical nature of the acyl binding site of an enzyme often include an investigation of the catalyzed hydrolysis of ester-substrates of varying acyl carbon chain length. A number of natural or synthetic triacylglycerols has also been used in studies of the lipolytic activity of different lipases. The effect of carboxylic (fatty) acid chain length on the lipolytic activity of rat lingual lipase has been tested using rat milk fat as

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PII S1381-1177(96)00002-1

substrate. The rat lingual lipase preferentially released the medium-chain free fatty acids (FFAs) (73 mol% of C8:0, C10:0 and C12:0 FFAs) [1,2]. Liao et al. [3] used various preparations of rat lingual lipase (whole homogenates of lingual serous glands or partially purified enzyme), and found that the hydrolysis of medium-chain triglycerides (e.g. trioctanoin) proceeded at rates 5–7 times faster than that of long-chain triglycerides. Roberts et al. [4] reported that the specific activity against tributyrin was approximately 5–10 times greater than that against triolein. DeNigris et al. [5] reported that the rate of hydrolysis of tricaprylin (C8:0) was 7.8- and 1.6-fold higher than that for hydrolysis of triolein (C18:1), catalysed by rat and mouse lingual lipase, respectively. For the human lingual lipase, Liao et al. [6] found that the lipase in human gastric aspirates hydrolyzed medium-chain triglycerides at a 5–7-fold higher rate than for long-chain triglycerides.

However, the pregastric lipase from ruminants preferentially released short-chain FFAs from milk, naphthol esters and synthetic triglycerides. Edwards-Webb and Thompson [7] used bovine milk as substrate, and found that the rate of calf pregastric lipase catalysed release of short- and medium-chain fatty acids (C4:0–C12:0) was much higher than the release of long-chain fatty acids, with a maximum rate occurring with butyric acid, C4:0. Hamilton and Raven [8] studied the relative activity for hydrolysis of triglycerides of different carbon chain length (from tributyrin to triolein) and found maximum activity against tributyrin. Purified lamb pregastric lipase was found by d'Souza and Oriel [9] preferentially to hydrolyze short-chain monoacid triglycerides (C4:0 and C6:0).

Although lipases are more active at a lipid–water interface, not all the factors which trigger their activation are known. Water-soluble substrates eliminate surface effects that may cause complications in investigations of the effect of alteration of the acyl carbon chain length of oil-phase substrates during their lipase-catalyzed hydrolysis.

2. Experimental

2.1. Materials and preparation of solutions

The enzyme preparation supplied by New Zealand Rennet was extracted from the tongue and epiglottis of suckling lambs. The preparation had been filter-sterilized and freeze dried and was supplied without the lactose extender of the standard commercial product.

The following were Sigma products: 4-nitrophenyl-acetate (PNPA), -propionate (PNPP), -butanoate (PNPB), -pentanoate (PNPPe), -hexanoate (PNPH), -octanoate (PNPO), -decanoate (PNPDe), and -dodedecanoate (PNPDo); tributyrin (C4:0), tricaproin (C6:0), tricaprylin (C8:0), and tricaprin (C10:0); Bis–Tris Propane, lecithin and sodium caseinate.

2.2. Enzyme processing

The processing of the commercial enzyme extract to produce the fraction with high activity against tributyrin has been described previously [10]. Briefly, the purification was directed towards the removal of inactive and non-protein components and was carried out by suspending the commercial product in 50 mM Tris/HCl buffer, pH 8.0, which was then loaded onto a fast flow Sepharose-Q anion exchange column at 4°C. Elution with 50 mM Tris/HCl yielded a non-bound fraction F1. Elution with 50 mM acetate/HCl buffer, pH 4.6, yielded fraction F2 with high activity against PNPA and very low activity against tributyrin. Final elution with 50 mM Tris/HCl, pH 8.0/1 M NaCl yielded fraction F3. Dialysis and lyophilization of fractions F2 and F3 yielded pale tan powders which were shown by Lowry protein assays to be composed of $\approx 100\%$ protein.

The specific activity of fraction F3 lipase was 2.1 μmol min^{-1} mg^{-1} (8.6 mM tributyrin, pH 6.5, 40°C) and 0.135 μmol min^{-1} mg^{-1} (1 mM PNPA, pH 7.2, 37°C; $\epsilon_{400nm} = 10.5 \cdot 10^3$ M^{-1} cm^{-1}). The specific activity of fraction F2 es-

terase was 0.514 μmol min^{-1} mg^{-1} (1 mM PNPA, pH 7.2, 37°C; $\epsilon_{400nm} = 10.5 \cdot 10^3$ M^{-1} cm^{-1}).

We have shown [11] that preheat treatment of fraction F3 at pH 7.2, 50°C for 15 min effectively removed its lipase activity against tributyrin, but there remained activity against PNPA. We have attributed this activity to a fraction F3 esterase component, and have further shown that the esterase components in fractions F2 and F3 are not identical [11].

The very small amount of activity against tributyrin in fraction F2 was similarly removed by preheat treatment for 10 min at 50°C. This procedure had little effect on the esterase activity of fraction F2 against PNPA [11].

2.3. Measurement of the rate constant of hydrolysis of 4-nitrophenyl alkanoates

The activities of three different enzyme fractions were monitored: fraction F3, preheated fraction F3 and preheated fraction F2. All kinetic runs on any one enzyme fraction were determined using the same stock solution. The stock solutions of the 4-nitrophenyl ester-substrates of different alkanoate carbon chain length were prepared by dissolving PNPA, PNPP, PNPB, PNPPe, PNPH, PNPO, PNPDe, PNPDo in dried acetonitrile (10 ml). The concentration of PNPA, PNPP, PNPB, PNPPe, PNPH and PNPO was 5.07 mM, and that of PNPDe and PNPDo was 0.79 mM.

The enzyme solution (50 μl) was added to a solution of Bis–Tris Propane buffer (3 ml, pH 7.2, 37°C) and equilibrated to 37°C. An aliquot of substrate solution (5 μl) was then added and the pseudo first-order rate constant of hydrolysis was obtained from measurement of the change in absorbance at 400 nm until the reaction had reached completion. The rate constant of background hydrolysis was also obtained from measurements in a solution of 3050 μl buffer and 5 μl substrate stock solution. The concentration of enzyme used to catalyze the reactions was adjusted so that the observed rate constants of hydrolysis lay within the range $1 \cdot 10^{-2}$–$5 \cdot 10^{-4}$ s^{-1}. All observed data were corrected for the rate constant of background hydrolysis and brought to unit concentration of added enzyme. The rate constants of hydrolysis by fraction F3 were also multiplied by a factor of 1.13 in order to compensate for enzyme inactivation during preheat treatment [11].

2.4. Measurement of the k_{cat} for hydrolysis of monoacid triglycerides

The initial rate of hydrolysis of each lipid substrate was assessed over a range of (normally ten) concentrations using a standard emulsifier base. This base was typically prepared by dissolving 400 mg lecithin and 2.4 g sodium caseinate in 300 ml H_2O, dispersing these emulsifiers by stirring, and then making the volume up to 400 ml. This relatively high concentration of lecithin was employed in order to enhance emulsification of the more concentrated hydrophobic substrates (e.g. 22 mM tricaprin and 45 mM tricaprylin).

In a typical run, the substrate was weighed into a titrator cup, the emulsifier was added and the mixture was sonicated at 35°C. The emulsion so formed was then placed in a thermal jacket in the autotitrator and stirred continuously throughout the subsequent titration. The final temperature and pH adjustments were made in situ.

The initial rate of hydrolysis of each lipid at each concentration was assayed by pH stat (pH 6.5) at 35°C. At zero time a 100 μl (or 200 μl for slow reactions) aliquot of enzyme solution (5.2 mg ml^{-1}) of fraction F3 was added and the reaction was monitored by titration of released carboxylic acid with standardised NaOH (typically 0.01 M).

The initial rate data set for each lipid was fitted with a hyperbolic curve using the Marquardt–Levenberg algorithm within Sigmaplot™ to yield a Michaelis–Menten plot. The k_{cat} values thus derived refer to the moles of substrate completely hydrolysed. The use of k_{cat} was

preferred to that of V_{max} since the absolute concentration of enzyme was unknown. The values of K_m obtained for hydrolysis of the lipids were 0.6 mM (C4:0), 2.7 mM (C6:0), 7.9 mM (C8:0) and 8.1 mM (C10:0).

3. Results

3.1. Effect of alkanoate carbon-chain length

Fig. 1 describes the effect of changing alkanoate carbon chain length on the rate constant of hydrolysis of the 4-nitrophenyl esters catalysed by lipase component F3 (upper plot) and esterase components F2 and F3 (lower plot), respectively. The pseudo first-order rate constants of hydrolysis of the 4-nitrophenylalkanoates by unheated F3 were corrected for the contribution of the rate constants of hydrolysis by preheated F3 (using the same mass of added enzyme) in order to obtain the contribution due only to the F3 lipase component. The rate constants of hydrolysis of the esters catalysed by preheated fractions F3 and F2 were corrected for the background rate constant of hydrolysis of these esters. All of the enzyme components exhibit maximum activity for an alkanoate ester of carbon chain length equal to 10, that is against PNPDe.

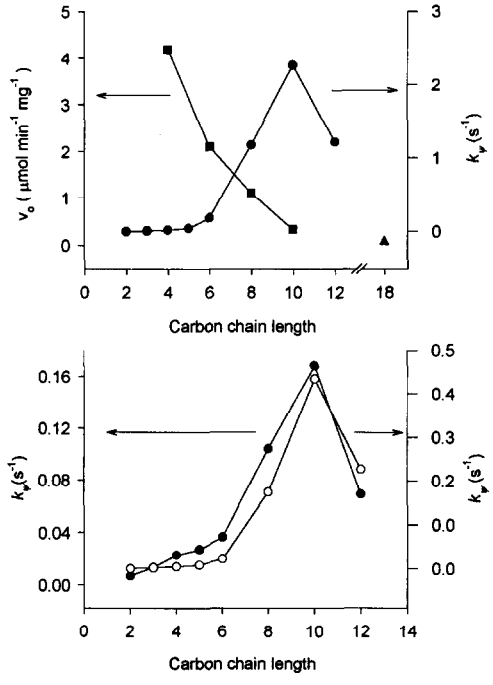

Fig. 1. Upper plot: Activity of fraction F3 lipase towards monoacid triglycerides, ■, at pH 6.5, 35°C and 4-nitrophenylalkanoates, ●, at pH 7.2, 37°C. The value for the rate of hydrolysis of triolein [10] is represented by ▲; lower plot: activity of fraction F3 esterase, ○, and of fraction F2 esterase, ●, towards 4-nitrophenylalkanoates at pH 7.2, 37°C.

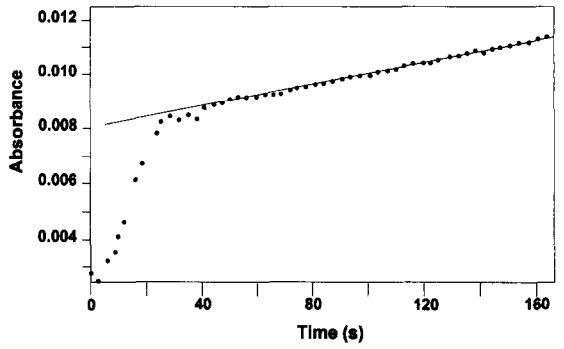

Fig. 2. The burst effect for hydrolysis of PNPDe catalysed by fraction F3 lipase at pH 7.2, 37°C.

3.2. Effect of monoacid triglyceride carbon chain length

Fig. 1 (upper plot) also shows the effect of increasing fatty acid carbon chain length on the values of k_{cat} for hydrolysis of the monoacid triglycerides catalysed by lipase component F3. Also given is the value for k_{cat} for hydrolysis of triolein obtained using a more concentrated emulsion of substrate and a sensitive radiochemical assay [10]. The initial rates are seen to decrease approximately 2-fold for each additional pair of carbon atoms in the fatty acid chain.

3.3. The burst effect

4-Nitrophenyl decanoate was the preferred substrate for catalysis by all three enzyme fractions. Initially, when setting up the experimental protocol, a relatively high concentration of en-

zyme (unheated fraction F3, $1.6 \cdot 10^{-2}$ mg ml^{-1}) was used in the assay, and a burst of production of the 4-nitrophenolate anion was observed (Fig. 2) in the pre-steady state. Under these conditions, the slope of the linear portion cannot be considered as the initial rate of hydrolysis. However, when the concentration of enzyme was decreased to $1.0 \cdot 10^{-1}$ $\mu g\ ml^{-1}$, the burst phenomenon was eliminated and the initial rate of hydrolysis was then able to be determined on a routine basis.

4. Discussion

4.1. Effect of alkanoate carbon-chain length

A series of 4-nitrophenylalkanoate esters has been used to assist in characterization of the active site of the lamb pregastric enzymes. The effect of increasing the carbon chain length of the alkanoate not only changes the hydrophobic nature of the ester, but also changes the size and shape of the substrate. All the substrates were used at a concentration below their critical micelle concentration, in order to avoid self micellization. Since saturation kinetics were not then applicable, all rate data were therefore obtained using an integrated rate equation.

The activity–carbon chain length profile for the F2 esterase component (Fig. 1, lower plot) shows less variation in the rate constant of hydrolysis as the carbon chain lengthens than does either the esterase (Fig. 1, lower plot) or the lipase component in fraction F3 (Fig. 1, upper plot). The relative range of rate constants for the F2 esterase, F3 esterase and F3 lipase components were 250-, 630- and 1250-fold, respectively. Although the rate constant of hydrolysis of PNPA catalysed by F2 esterase remains much greater than those catalyzed by either the F3 esterase or the F3 lipase components, the rate constant of hydrolysis of PNDDe catalysed by F2 esterase is only 38% of the activity of the F3 esterase component and 6% of the activity of the F3 lipase component.

The results in Fig. 1 show that the lamb pregastric lipase, and both esterase components (F2 and F3) have similar profiles for chain length against activity, with maximum activity occurring against PNPDe (C10). This coincidence may be the result of locking the substrate into an active site of similar size and orientation for all three enzymes. More information about the amino acid residues in the active site is necessary to resolve this question. However, the profiles for chain length against activity do supply some fingerprint information about the active site of the various enzyme components.

A study of the hydrolysis of α-naphthyl esters and monoacid triglycerides catalyzed by lamb pregastric lipase has been made by d'Souza and Oriel [9]. The maximum activity of hydrolysis for α-naphthyl alkanoate esters was found to occur at an alkyl carbon chain length equal to six (α-naphthylcaproate) and for hydrolysis of monoacid triglycerides at an acyl carbon chain length equal to four (tributyrin).

O'Connor and Wallace [12] explored the esterase acyl binding site of bile-salt stimulated human milk lipase by investigating the enzyme catalysed hydrolysis of a series of 4-nitrophenylalkanoates and of 4-nitrophenylbenzoate and methylbenzoate. They found that the rate of catalysed hydrolysis increased with increasing carbon chain length and proposed that the acyl binding site had the shape of a long narrow pocket with a width of 0.2 nm. The pocket could then accommodate hydrophobic binding of a benzoate ring with an edge-on orientation or a single *n* fatty acid in an extended conformation.

However, such a proposal is not adequate to explain the results thus far presented for activity of lamb pregastric lipase, for which there is a carbon chain length difference for appearance of maximum activity depending on whether the substrates are α-naphthyl- or 4-nitrophenyl-alkanoates. However, we show below that these two results, which at first sight appear to be contradictory, are probably self consistent. Both substrates, α-naphthylcaproate and 4-

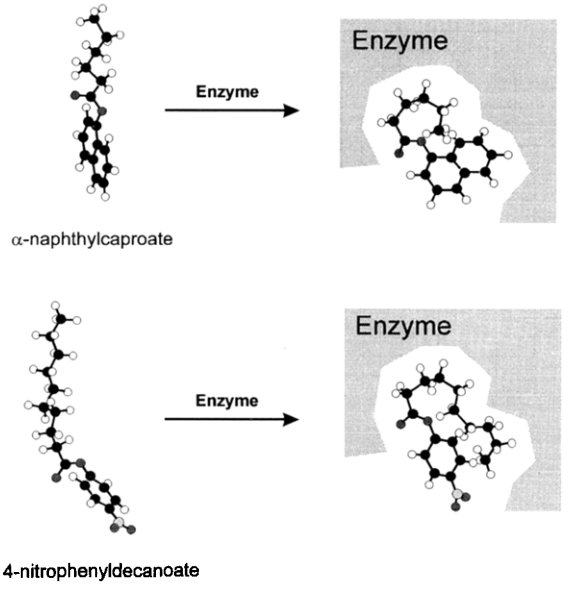

Fig. 3. Diagram illustrating the possible conformation of α-naphthylcaproate (lower) and 4-nitrophenyldecanoate (upper) binding to lamb pregastric lipase. Left: the extended conformation of the free molecules; right: possible buckled conformation of the substrates within the active site.

nitrophenyldecanoate, contain a total of 16 carbon atoms. In their extended forms they appear to have different conformations, but model building shows that the alkyl chain of the phenyldecanoate ester can be folded back towards the phenyl ring in such a way that the buckled structure is almost superimposable on the buckled structure of the naphthylcaproate ester in which the alkyl chain is folded back towards the naphthyl rings. (The contribution of the $-NO_2$ group to the overall size of the molecule is ignored in this suggestion, since it is assumed that it will be positioned at the outer extremity of the active site.) Fig. 3 represents these suggestions.

4.2. *Effect of carboxylic acid chain length of monoacid triglycerides*

For each additional pair of carbon atoms in the carboxylic acid chain in the monoacid triglycerides, the value of k_{cat} decreases by at least a two-fold factor. This factor is less than that observed by d'Souza and Oriel [9]. They measured a decrease of more than three-fold in the rate of hydrolysis between tributyrin and tricaproin, and were unable to detect any activity for longer chain lipids.

However, their measurements were made using a turbidometric technique where the loss in absorbance at 340 nm was a measure of the amount of lipid that was hydrolyzed. The conditions of their experiment were pH 6.0, temperature not stated, and only a single (3 mM) concentration of lipid was used. We have found that K_m increases as k_{cat} decreases. The values of K_m for C8:0 (7.9 mM) and C10:0 (8.1 mM) are above the concentration used by d'Souza and Oriel [9]. Therefore, under their conditions, as the chain length increases, the observed rate changes from nearly zero order to first order in substrate. Thus, as the chain length is increased, measured rates of hydrolysis lie progressively lower with respect to the plateau region and k_{cat} of the appropriate Michaelis–Menten hyperbola.

The triangle in Fig. 1 (upper plot) represents the value of k_{cat} for hydrolysis of triolein (C18:1) derived under more concentrated conditions of substrate in a different emulsion system and using a sensitive radiochemical assay technique. (The value of K_m was determined as 9 mM under the experimental conditions.) This measured rate is smaller yet again than that for tricaprin (C10:0) but given the differences in the assay conditions and the unsaturated nature of the substrate compared with the saturated lipids used in this investigation, further quantitative comparison is not justified.

4.3. *Commercial applications*

Harper and Gould [13] found multiple pH optima for the pregastric lipases of three species (for the calf enzyme pH 5.3, 6.1 and 7.5; for the kid enzyme pH 5.5, 6.2 and 8.6; and for the lamb enzyme 5.9 and 6.6) using milk-fat as substrate. These results suggest that the commercial extracts used in cheese production con-

tain multiple active components, but no reports have been made on separation of the species in calf and kid. However, it is well known that the individual species' extracts play a critical role in the development of characteristic flavors and *picante* of Italian style cheeses [13–15].

This present study has identified some properties of substrates which are desirable for hydrolysis by the active components in the lamb extract. Studies are in hand to isolate the active components in the extracts from calf and kid and to characterize similarly their substrate selectivity. These comparative results should assist in providing an explanation for the different properties of ruminant pregastric extracts used in cheese making.

Acknowledgements

We acknowledge financial assistance from the Research Committee of the University of Auckland, New Zealand Lottery Science and the New Zealand Agricultural & Marketing Research Development Trust. We are especially grateful to Dr K.W. Turner and New Zealand Rennet for the kind gift of pregastric enzyme and continued interest and to Mr P.A.G. Butler for his technical expertise.

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