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Lamb pregastric lipase catalysed hydrolysis of 1,2,3-tri[(*cis*)-9-octadecenoyl]glycerol: temperature, pH and solvent isotope effects

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Abstract

Lamb pregastric lipase, extracted from the tongue and epiglottal region of lamb, has been partially purified and used to catalyse the hydrolysis of (9–10 ^{3}H)(1,2,3-tris-[(*cis*)-9-octadecenoyl]glycerol) over the pH range 5.50–7.50 and temperature range 20.0–40.0°C. Michaelis–Menten plots were constructed for each reaction condition, and allowed evaluation of K_m and k_{cat}. The values of k_{cat} have been fitted to a three-dimensional activity profile. The optimum pH for reactivity was 6.3 ± 0.3 and the optimum temperature 32 ± 3°C. Under optimum conditions the values of k_{cat} and K_m were 0.073 μmol · min^{-1} · mg^{-1} and 9 mM, respectively. The reaction has also been studied in D_2O as solvent at pD = 6.50 and T = 30.0°C. The kinetic isotope effects were 1.46 and 1.31 for the rate determining acylation and deacylation steps, respectively. The activity of the enzyme was inhibited by the presence of the bile salt, sodium taurocholate.

Keywords: Lamb pregastric lipase; Solvent isotope effects; Hydrolysis of triolein; Enzymic catalysis; Bile salt inhibition

1. Introduction

Pregastric lipase is an enzyme of oral and pharyngeal origin in ruminants. Studies on localisation of the source of the enzyme have shown a similar distribution to that found for lingual lipase in rodents. Hamilton and Raven [1] found that calf pregastric lipase catalysed short- and medium-chain triglycerides more readily than long-chain ones, with very low activity against triglycerides composed of fatty acids of chain length greater than 16. A similar study undertaken by d'Souza and Oriel [2] on the activity of lamb pregastric lipase failed to detect activity with triglycerides composed of fatty acids of chain length greater than 8.

Studies with crude pregastric lipase have generally supported Ramsey et al.'s [3] findings that it consists of more than one protein. Harper and Gould [4] 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 pH 5.9 and 6.6) using milk-fat as substrate. O'Connor et al. [5] found that crude calf pregastric lipase had maximum activity at pH 6.9 against the lipid substrate, tributyrin, but at pH 7.5–8.5 against the ester substrate, 4-nitrophenylacetate, PNPA. This difference was accounted for by hypothesising the presence of at least two enzymes, an esterase

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showing dominant activity against the ester, and a lipase accounting with a different pH optimum against tributyrin. Crude lamb pregastric lipase showed maximum activity against tributyrin at pH 6.6, and at pH 6.5–6.8 against PNPA. Richardson and Nelson [6] found a single pH optimum at pH 5.5, for all of calf, kid, and lamb pregastric lipases, using tributyrin or milk-fat as substrates.

Studies with purified calf pregastric lipase [7] have shown the enzyme to have a wide pH optimum range of 4.5 to 6.5. d'Souza and Oriel [2] found the pH optimum for purified lamb pregastric lipase against α-naphthyl butyrate to be between pH 5.5 and 6.0.

Harper and Gould [4] found that with milk-fat as the substrate, crude calf pregastric lipase had a temperature optimum of 28–30°C, and lamb and kid pregastric lipase had an optimum temperature of 32–34°C. O'Connor et al. [5] found that crude calf pregastric lipase had a temperature optimum of 37.5°C against PNPA, while crude lamb pregastric lipase had a temperature optimum of 30.0°C. d'Souza et al. [2] showed that purified lamb pregastric lipase was rapidly inactivated at temperatures above 30°C.

Although lamb pregastric lipase has been used for many years in industry, especially as a flavour enhancer of cheeses, very little is known about its activity against hydrophobic (long-chain) lipids, the site of its activity for hydrolysis of fats in the digestive tract of the lamb, or its mechanism of action.

We have discovered that the lamb pregastric lipase preparation, commercially available from the New Zealand Rennet Co. Ltd, Eltham, New Zealand, contains at least two active enzymes. Therefore the study of this enzyme mixture would have led to confusing results. One of the active components was therefore separated from the mixture and partially purified. Although neither the separation or purification have been optimised, the fraction used in the studies which follow was homogenous and contained $\approx 100\%$ protein by weight. This fraction was active against esters and both short and long chain triglycerides. The account which follows focuses upon the determination of a lamb pregastric lipase catalysed hydrolysis of 1,2,3-tris[*cis*-9-octa-decenoyl]glycerol (triolein) and the deuterium isotope effect of this reaction. A brief investigation has also been made on the effect on the activity of the bile salt, sodium taurocholate. As a result of these studies, considerable progress has been made towards defining details of the conditions for optimum activity. Additionally, restrictions have been placed on the location in the digestive tract, of this ruminant, where this enzyme may be useful.

2. Experimental

2.1. Materials and preparation of solutions

The enzyme preparation supplied by the New Zealand Rennet Co. Ltd was extracted from the tongues and epiglotti of suckling lambs. The preparation had been filter-sterilized and freeze dried and was supplied without the lactose extender of the standard commercial product. Further partial purification was directed towards removal of inactive and non-protein components and concentration of the lipase component for kinetic studies.

The crude raw product was suspended in 50 mM Tris/HCl buffer, pH 8.0, allowed to equilibrate and then centrifuged, and the supernatant was loaded onto a column containing a fast-flow Sepharose-Q anion exchange phase. Elution with 50 mM Tris/HCl buffer, pH 8.0, yielded the non-bound material, which was discarded. Elution with 50 mM acetate/HCl buffer, pH 4.6 yielded a fraction with high esterase and low lipase activity. Final elution with 50 mM Tris/HCl, pH 8.0/1 M NaCl yielded a fraction, from which, after dialysis and lyophilization, a pale tan powder was isolated with relatively high activity against the short chain triglyceride, tributyrin, and also activity against PNPA.

The protein content of this extract was $100 \pm 5\%$ (cf. $62 \pm 3\%$ for the raw precursor); the esterase activity was 45 μmol·min^{-1}·mg^{-1} (1 mM PNPA, pH 7.2, 37.5°C) and the lipase activity was 1.8 μmol·min^{-1}·mg^{-1} (20 mM tributyrin,

pH 7.2, 37°C). Polyacrylamide gel electrophoresis with protein mass standards identified the molecular weight range of the partially purified lipase fraction to be within the range 44–68 kDa.

Triolein (1,2,3-tri[(*cis*)-9-octadecenoyl]glycerol), >99%, was from Sigma and (9–10 ^{3}H) triolein, 1 mCi (37 nmol, carrier free) was purchased from Amersham. Immediately upon receipt of the radioactive lipid, a stock solution in benzene was prepared which contained 0.2 $g \cdot ml^{-1}$ triolein spiked with ^{3}H-triolein, final activity 46.3×10^{-1} Bq s^{-1}.

Bis[2-hydroxyethyl]iminotris [hydroxymethyl]methane (Bis–Tris) and 1,3-bis[tris(hydroxymethyl)methylamino]propane (Bis–Tris–Propane) were from Sigma. Deuterated Bis–Tris (D-Bis–Tris) was prepared by exchanging Bis–Tris in D_2O four times until the NMR spectrum showed no further decrease in the amine hydrogen signal.

4-Nitrophenylacetate, sodium taurocholate (99%) and lysolecithin were from Sigma and bovine serum albumin from Serva.

Mathematical data processing was carried out using Sigmaplot software, version 5.0 (Jandel Scientific) on an IBM compatible 80486 personal computer and the contour plot was generated from the Sigmaplot interpolated data matrix using MathCad 4.0 for Windows (MathSoft Inc.).

The following solutions were prepared. Lamb pregastric lipase, 4 $mg \cdot ml^{-1}$ in Milli-Q water or D_2O; 0.2 M Bis/Tris/HCl buffer at pH 5.50, 6.00 and 6.50; 0.2 M Bis–Tris–Propane/HCl buffer at pH 7.00 and 7.50; 0.2 M D-Bis–Tris/HCl buffer at pD 6.50; quench buffer, 0.1 M sodium borate/sodium carbonate, pH 10.5; bovine serum albumin, 1% and 4% solutions in the appropriate buffer; lysolecithin, 3 $mg \cdot ml^{-1}$ in chloroform containing 0.1% Milli-Q water or D_2O; organic reagent, $MeOH:CHCl_3:C_7H_{16} = 1.41:1.25:1$ (v/v/v).

2.2. Preparation of the substrate emulsion

Up to 10% hydrolysis of the stock solution of ^{3}H-triolein occurred during storage. Before commencing the kinetic investigation the substrate was purified by chromatography on Bond-Elut aminopropyl columns using the method of Kaluzny et al. [8]. Purified triolein solution (various amounts to yield a final concentration within the range 0.63 to 25.2 mM) and a volume equivalent amount of lysolecithin were placed in a 1.5 ml Eppendorf tube and dried under a stream of nitrogen. Buffer solution (630 μl) and bovine serum albumin solution (120 μl, 1%) were added and the mixture was sonicated in an ice bath for 4 min in bursts of 30 s on, 30 s off. Bovine serum albumin (150 μl, 4%) was then added and the whole mixed by inversion.

2.3. Preparation of the reaction mixture

The substrate emulsion (450 μl) was transferred to a 1.5 ml Eppendorf tube and placed in a water bath at the required temperature (vial A). To another Eppendorf tube was added the lipase solution, typically 40 μl, followed by buffer solution, typically 360 μl, and this tube was also placed in the water bath at the required temperature (vial B). After 5 min, 400 μl from vial A was added to vial B, the whole vortexed for 5 s, and immediately returned to the water bath. This moment was considered to be zero reaction time.

3. Determination of reaction kinetics

3.1. Quenching procedure and extraction of product from reactants

At various times, 60 μl of the reaction mixture was removed and mixed with 250 μl of quench buffer in an Eppendorf tube. The organic reagent (1 ml) was then added, the mixture was vortexed for 15 s and placed in a temperature controlled centrifuge set at 20°C. A constant extraction temperature was necessary to prevent variations in extraction efficiency. The tubes were then centrifuged for 10 min at 2900 g. A 100 μl sample from the top (aqueous) layer was added to 3 ml Aquassure scintillation fluid (NEN) and the radioactiv-

ity was determined on an LKB Wallac 1219 liquid scintillation counter.

A control sample, prepared by adding substrate emulsion (30 μl) and Bis–Tris/HCl buffer (30 μl) to the quench buffer (250 μl), and a background sample, prepared by adding Bis–Tris/HCl buffer (60 μl) to the quench buffer (250 μl) were also included in the experimental protocol. Both were subjected to the standard extraction procedure.

3.2. Michaelis–Menten kinetics

The Michaelis–Menten treatment [9] of enzyme kinetics assumes the reversible formation of an enzyme–substrate intermediate followed by a first-order decomposition of complex to product.

$$E + S \underset{}{\overset{k+1}{\rightleftharpoons}} ES \xrightarrow{k+2} E + P \qquad (1)$$

Here, E, S and P are the enzyme, substrate and product, respectively.

The values of V_{max} (or k_{cat}), the rate for breakdown of the Michaelis–Menten complex, and the rate for the formation step, V_{max}/K_m, may be derived from appropriate curve fitting of the plot of ν_0, the initial velocity, against [S].

3.3. Efficiency of extraction of oleic acid

The efficiency of extraction of oleic acid was determined as follows. A sample of ^{3}H-triolein was completely hydrolysed in concentrated NaOH to obtain a pure sample of radioactive oleic acid (m.p. 13°C). Emulsions of ^{3}H-oleic acid, to cover the range of concentrations of 5–100% hydrolysis of triolein, were then prepared as described above for triolein, and subjected to the standard extraction procedure.

Control samples, prepared by adding 60 μl aliquots of oleic acid emulsion directly into scintillation vials containing scintillation fluid (3 ml), were also included in the reaction protocol.

The efficiencies for extraction of oleic acid were determined for pH 5.50, 6.50 and 7.50, and also for two mixtures of radioactive oleic acid and unlabelled triolein in the ratio of 1:1 and 1:3 at pH 6.50. All extractions were carried out in triplicate.

3.4. Effect of temperature and pH

The general procedure for determining the lamb pregastric lipase catalysed hydrolysis of triolein is outlined above. Reactions were carried out at pH 5.50, 6.00, 6.50 (using Bis–Tris buffer, 0.2 M) and pH 7.00 and 7.50 (using Bis–Tris–Propane buffer, 0.2 M), and at temperature of 20.0, 25.0, 30.0, 35.0 and 40.0°C. For each of the 25 reaction conditions, the initial rates of hydrolysis against at least five different triolein concentrations (typically 1.26, 1.89, 2.52, 6.30, 12.6 mM) were determined (in duplicate), so that Michaelis–Menten plots could be constructed and the k_{cat} and K_m values for every condition determined. The reproducibility of duplicate runs was generally within ±5%. Each reaction was initiated by adding 200 μl of substrate emulsion to an equal volume of enzyme solution (4 mg·ml^{-1}), and aliquots were removed for analysis at one minute intervals for the first six minutes of the reaction.

3.5. Mathematical processing

The data points within each pH – temperature condition were fitted to functions of two sorts. The original data for the Michaelis–Menten plot were fitted to a hyperbolic curve using the Marquardt–Levenberg algorithm within Sigmaplot. The data were also plotted as reciprocals in the form of a Lineweaver–Burk plot to which a linear least-squares regression fit was made. For perfect data, the two procedures will lead to identical results. The evenly weighted method was preferred and the Michaelis–Menten parameters used below were taken directly from the hyperbolic least-squares fit.

The total k_{cat} data set was collated for processing into the kinetic surface plots. Third order polynomial fits were used for slices of constant pH and constant temperature and then the surface net was constructed using the resultant polynomials. The original 25 (5×5) Michaelis–Menten values

generated 441 (21×21) values, giving a finer grain to the plot. This interpolation procedure was primarily introduced to suppress artifacts produced by the operation of the Sigmaplot surface net construction algorithm and it had a secondary effect of smoothing the data a little. This enlarged data set also formed the basis for the MathCad calculation of the contour plot, and thus the contour plot exactly maps the surface net plots.

3.6. Determination of the solvent isotope effect

Initial rates for the lamb pregastric lipase catalysed hydrolysis of triolein were determined (in duplicate) for a range of substrate concentrations (0.63–25.2 mM), using D_2O and H_2O as solvents. Aliquots (60 μl) were taken at one min intervals for a total reaction time of six min. The reactions were carried out at 30°C and at pH = pD = 6.50. Thus it was ensured that all rate measurements were related to identical acidities. The initial rates observed were used to construct Michaelis–Menten plots for the two reaction systems, and the values of k_{cat} and K_m were determined.

The pH value of 6.50 lies within the plateau range for maximum activity of lamb pregastric lipase, so that equal fractions of active lipase would have been present in the two solvents H_2O and D_2O. (The inflexion point on pL/rate profiles commonly shifts to the basic by up to 0.5 units in D_2O.)

A study of the measurement of acidity in deuterium oxide solutions has been undertaken by Glasoe and Long [10]. They found that the pH meter reading was 0.40 pH units lower in D_2O solution than in H_2O solution and that the relationship between pH and pD may be represented by Eq. 2

$$\text{pD} = \text{pH meter reading} + 0.4 \quad (2)$$

where the pH meter reading is obtained with an apparatus standardised to read pH in H_2O solutions. The values of pD reported in this study are those obtained after taking this relationship into account.

The D-Bis–Tris/DCl buffer solution (0.2 M) was prepared by dissolving D-Bis–Tris in D_2O and adjusting the pD to 6.50 by addition of DCl in D_2O.

3.7. Effect of taurocholate

The activity of the enzyme was assayed (in duplicate) against triolein (3.78 mM), in the presence of a range of taurocholate concentrations (0–20 mM). The enzyme solution was prepared by adding taurocholate solution and Milli-Q water (combined volume of 40 μl) to the enzyme stock solution (160 μl). Substrate emulsion (200 μl) was added to the enzyme solution to initialise the reaction. Aliquots (60 μl) were removed for analysis at one minute intervals for the first six min of the reaction. All reactions were carried out at pH 6.50 and 30.0°C.

4. Results

4.1. Extraction efficiency of oleic acid

Under all reaction conditions of varying pH, oleic acid concentration and ratio of triolein:oleic acid the extraction efficiency was 58 ± 1%.

4.2. Lamb pregastric lipase catalysed hydrolysis of triolein

The k_{cat} values obtained for each reaction condition were first fitted by third order curves, and then to the three-dimensional profiles shown in Figs. 1 and 2.

The k_{cat} values forced to the 3-D plot showed reasonable agreement (typically within 10%) with the measured k_{cat} values, and the interpolated activity maximum was 0.073 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, at pH 6.1 and 34°C. The highest experimental value of k_{cat} was 0.077 $\mu mol \cdot min^{-1} \cdot mg^{-1}$, at pH 6.5 and 30°C. Unlike the single activity peak identified for the k_{cat} values, the K_m values, measured over the various reaction conditions, displayed no consistent trend. These

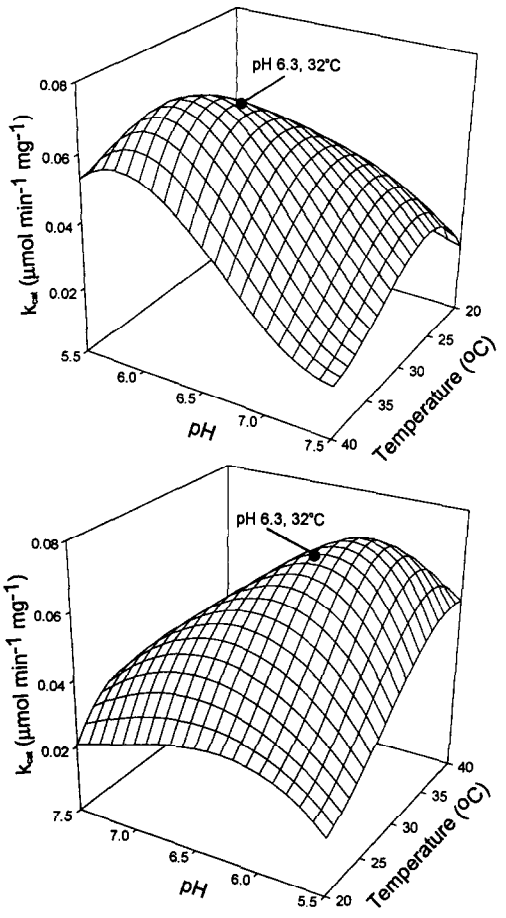

Fig. 1. The 3-D-kinetic surface plots for lamb pregastric lipase catalysed hydrolysis of triolein over the pH range 5.5–7.5 and temperature range 20–40°C (upper plot) and obverse view (lower plot). The maximum activity occurs at pH = 6.3 ± 0.3 and at 32 ± 3°C.

latter values lay within the range 7.42–10.66 mM. The value of K_m, at the experimentally determined maximum value of k_{cat}, was 9.03 mM.

The range of optimum conditions for lamb pregastric lipase catalysed hydrolysis of triolein was pH 6.3 ± 0.3 and 32 ± 3°C with an optimum $k_{cat} = 0.073 \pm 0.003$ μmol · min^{-1} · mg^{-1} and with $K_m = 9 \pm 1$ mM.

A different view of the experimental data is shown in Fig. 2 which depicts the contour lines of constant activity for the lamb pregastric lipase catalysed hydrolysis of triolein.

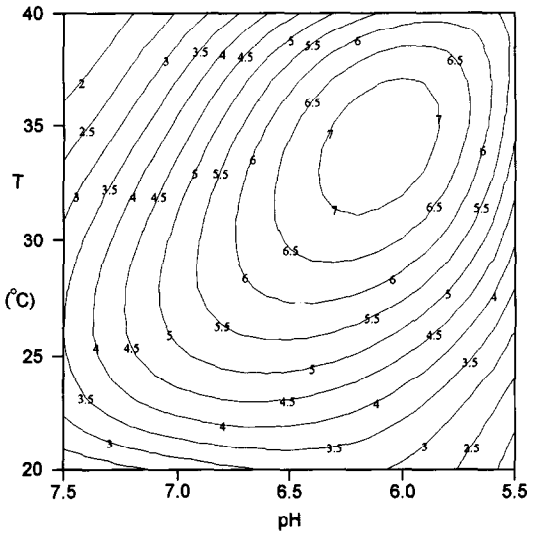

Fig. 2. Plot of temperature vs. pH showing contour lines of constant enzymic activity for the lamb pregastric lipase catalysed hydrolysis of triolein, using the data shown in Fig. 1. The region of activity within 10% of the optimum is relatively restricted. (A 100-fold scaling factor has been used on the contour lines in order to avoid the untidiness of leading zeros.)

4.3. The solvent isotope effect

Michaelis–Menten plots for the initial rates observed for the lamb pregastric lipase catalysed

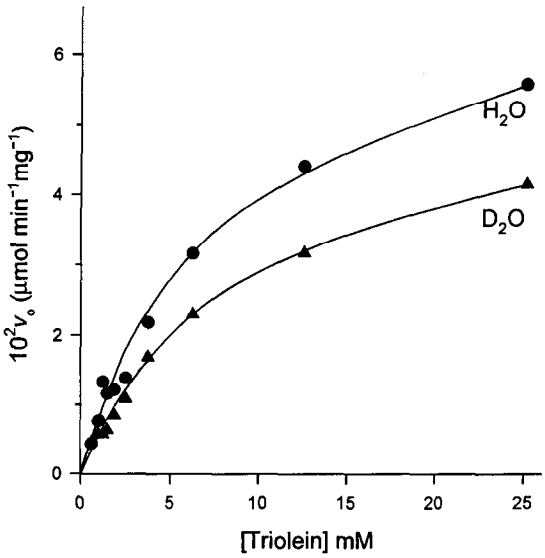

Fig. 3. Michaelis–Menten plots for the lamb pregastric lipase catalysed hydrolysis of triolein in H_2O and D_2O at pH = pD = 6.50, $T = 30$°C. The derived values of k_{cat} are 0.073 and 0.057 μmol · min^{-1} · mg^{-1} and of K_m are 9.0 and 10.1 mM, for reactions in H_2O and D_2O, respectively.

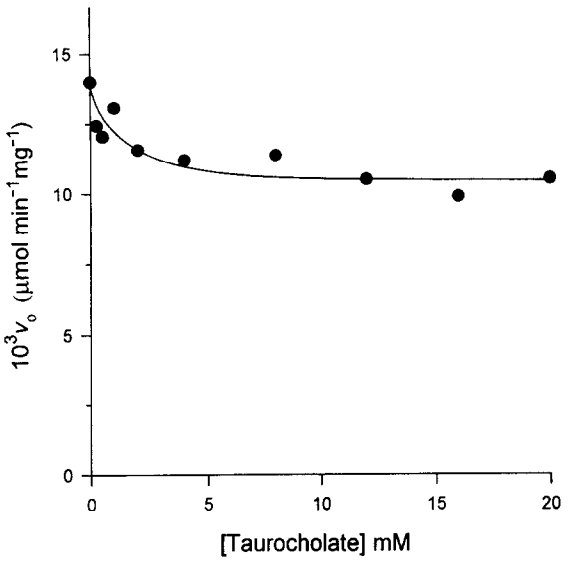

Fig. 4. The effect of taurocholate concentration on the initial rate of lamb pregastric lipase catalysed hydrolysis of triolein (3.78 mM) at pH 6.50 and $T = 30.0$°C.

hydrolysis of triolein using H_2O and D_2O as solvents are shown in Fig. 3. From these plots, values of k_{cat} equal to 0.077 ± 0.003 and 0.057 ± 0.008 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1} and of K_m equal to 9.0 ± 0.8 and 10.1 ± 0.6 mM, were determined for the reactions in H_2O and D_2O, respectively.

4.4. Effect of sodium taurocholate

The results for the initial rates of hydrolysis of the lamb pregastric lipase catalysed hydrolysis of triolein at various taurocholate concentrations are shown in Fig. 4. The initial rates decreased with increasing taurocholate concentration. The highest enzyme activity was observed in the absence of taurocholate.

5. Discussion

5.1. The reaction profile

The presence of only one activity peak within the measured temperature and pH ranges (Figs. 1 and 2) suggests that there is only one active lipase in the protein obtained from the purification process. The peak occurs at a slightly acidic pH (6.3 ± 0.3). This indicates that the enzyme is utilised before leaving the stomach of the lamb, but further investigation over a lower range of pH values is necessary before establishing whether activity continues under the very acidic conditions encountered within the stomach. The optimum pH for the enzyme catalysed hydrolysis of the long chain lipid, triolein, shows close agreement to that (pH 6.4 ± 0.3) [11] found with the same lipase component as used herein) for the hydrolysis of tributyrin, a short chain lipid, and to the pH optimum for the hydrolysis of the ester PNPA (pH 6.5–6.8) [5] (determined using the raw precursor). These similarities suggest that the same mechanism operates for a range of ester and lipid substrates.

However, since the maximum k_{cat} value obtained for the hydrolysis of triolein (0.073 ± 0.003 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1}) is significantly lower than that obtained [11] for the hydrolysis of tributyrin (2.6 ± 0.2 μmol $\cdot$ min^{-1} $\cdot$ mg^{-1}) there is a marked preference in substrate specificity for the short chain lipid. This preference is reflected in the values of K_m, equal to 9 ± 1 and ≤ 0.2 mM for the hydrolysis of triolein and tributyrin, respectively. The scatter observed in the experimentally determined values of K_m for the enzyme catalysed hydrolysis of triolein may arise, in part, from the relatively small concentrations of substrate used, compared with the value of K_m. Ideally, the substrate concentration should be at least 3–5 times that of K_m, but in this investigation the highest triolein concentration used was only 30% above the average K_m value. The limitation on the amount of substrate used was imposed by the amount of radioactively labelled triolein available.

In contrast to the contour lines of constant activity generated for the lamb pregastric lipase catalysed hydrolysis of tributyrin [11], which shows a very broad region of high activity surrounding the point of optimum activity (at pH 6.4, 43°C), the region of activity within 10% of the optimum value shown in Fig. 2 is relatively restricted.

5.2. Solvent isotope effect

At the present time, no sequence studies have been carried out on the amino acid composition of the active site of any pregastric lipase. d'Douza et al. [2], however, have determined the amino acid composition of lamb pregastric lipase and it contains 44 residues of Glu, 49 of Ser and 11 of His, all of which have the potential to participate in an acylation/deacylation mechanism, such as that proposed for the serine esterases, cholinesterase and acetylcholinesterase, and a serine protease, α-chymotrypsin [12] and for bile salt stimulated lipase [13].

Stout et al. [14] have suggested a means for determining the solvent isotope effect for rate determining acylation and deacylation steps. They measured the solvent isotope effects for the cholesterol esterase catalysed hydrolysis of 4-nitrophenyl-butyrate. In their analysis, they calculated the solvent isotope effect for the rate determining enzyme acylation from Eq. 3

$$^{D}(V_{max}/K_m) = (V_{max}/K_m)^{H_2O}/(V_{max}/K_m)^{D_2O} \quad (3)$$

and that for the rate determining deacylation step from Eq. 4

$$^{D}V_{max} = V_{max}^{H_2O}/V_{max}^{D_2O} \quad (4)$$

Substitution of the experimental values of V_{max} and K_m obtained in this investigation into Eqs. 3 and 4 yielded values for the solvent isotope effect for acylation and deacylation equal to 1.46 ± 0.08 and 1.31 ± 0.07, respectively.

We also showed that the conformation of the enzyme was not altered significantly when it was dissolved in D_2O. When the enzyme was first dissolved in D_2O and then tested against PNPA in H_2O it was found to exhibit the same activity after minutes only, or after many days, following the preparation of the enzyme solution. It is reasonable to assume that percolation of H_2O into the interstices of the protein would be slow, so exchange of the protein bound deuterium atoms for hydrogen would be too slow to cause the alteration in the conformation of the enzyme which would have been necessary to account for the difference in activity of the enzyme in the two different solvents.

Moreover, the equilibrium position for completion of the reaction showed the production of almost identical quantities of oleic acid, whether the solvent were H_2O or D_2O.

The effect of changing pD, on the initial rate of hydrolysis of triolein in D_2O, was similar to that observed for changing pH in H_2O (data not shown). The optimum reaction conditions were not altered, as might have been expected. This result demonstrates that the details of the reaction mechanism had not been altered and also shows that the values of k_{cat} and K_m were determined at optimum conditions for both solvents. Thus any difference in these values must be due to the solvent isotope effect and not to the fact that the reactions were undertaken at different points on the activity profiles.

The results obtained for the solvent isotope effects for the lamb pregastric lipase catalysed hydrolysis of triolein seem to indicate that in the rate-determining-step of the reaction mechanism the substituted atoms are not directly involved in the bond-breaking or bond-making process. This surprising result leads to the conclusion that a secondary kinetic isotope effect is operative and that further investigation is required before any details of the reaction mechanism for the enzyme-catalysed lipid hydrolysis may be derived.

The values of solvent isotope effects obtained for this enzyme are in marked contrast to the corresponding values obtained for bile-salt-stimulated human milk lipase, an enzyme utilised by infants for the digestion of the fats contained in milk. For this enzyme the calculated effects were 2.63 ± 0.11 for acylation and 3.14 ± 0.16 for deacylation [13]. These values are consistent with the participation of the hydrogen/deuterium atoms in a primary kinetic isotope effect and therefore very viable suggestions could be made with respect to the details of the reaction mechanism. The results gave specific evidence for the implication of serine and/or the imidazolium group of

histidine, both of which have been identified in the active site of the enzyme.

Before further progress can be made on the mechanism of activity of lamb pregastric lipase, information on the structure of the active site of the enzyme is required. In particular, its primary structure (amino acid sequence) needs to be determined so as to provide information to determine which amino acid residues are involved in the catalytic action of the enzyme.

5.3. *Effect of sodium taurocholate*

The results in Fig. 4 show that lamb pregastric lipase is slightly inhibited (up to 20%) by the presence of taurocholate.

Unfortunately, this result is not entirely conclusive, since the presence of bovine serum albumin in the reaction mixture may have masked any small fatty acid acceptor properties that the taurocholate might have been expected to exert. However, the absence of any bile salt stimulation, even in the presence of bovine serum albumin, is in marked contrast to the behaviour of bile salt-stimulated human milk lipase, whose activity was enhanced 150-fold in the presence of 10 mM taurocholate [15] under reaction conditions similar to these present ones. This evidence gives strong support to the supposition that the enzyme is not utilised for fat digestion in the lower part of the gut, in the region where the pancreatic enzymes (e.g. carboxylester hydrolase, an enzyme with similar bile-salt activation properties to bile salt-stimulated human milk lipase) come into play.

Further experiments will be required to determine the nature of the inhibition which the bile salt seemingly exerts.

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