Characteristics of Tributyroylglycerol Hydrolysis Mediated by a Partially Purified Lamb Pregastric Lipase

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ABSTRACT

A partially purified pregastric lipase from lambs was used as a catalyst for the hydrolysis of tributyroylglycerol. The reaction was followed by pH-stat autotitration of the released butyric acid. Integral values for pH and temperature were selected for determination of full Michaelis-Menten plots, and a kinetic surface was derived to characterize the activity of the enzyme preparation over a wide combination of these conditions. This surface enclosed a region of maximum activity centered on optimal pH (pH 6.4) and temperature (43°C), although optima were not sharply delineated. The activity in the surrounding region was generally high, and the value of the Michaelis-Menten constant was < 0.2 m*M*, indicating that tributyroylglycerol was generally a highly favored substrate.
(**Key words**: lamb, pregastric lipase, hydrolysis, tributyroylglycerol)

Abbreviation key: **bis tris propane** = 1,3-bis[tris-(hydroxymethyl)-methylamino]propane, K_{cat} = apparent turnover number, K_m = Michaelis-Menten substrate affinity constant, **4-NPA** = 4-nitrophenylacetate, **TBG** = tributyroylglycerol, **TOG** = trioleoylglycerol.

INTRODUCTION

Pregastric lipase from lambs is used for flavor and texture enhancement in the food processing industry, particularly for the modification of milk fat. The enzyme is primarily secreted from serous glands in the tongues of suckling lambs. The freeze-dried commercial enzyme preparation is manufactured by extraction from bulk quantities of the tongue root and epiglottis.

The commercial product is a mixture of many proteins, heme fragments, polysaccharides, and other remnants of the original biological matrix. Previous work has broadly characterized this crude mixed product against tributyroylglycerol (**TBG**), trioleoylglycerol (**TOG**), and 4-nitrophenylacetate (**4-NPA**) (13). This product contains at least two active enzymes with differing lipolytic and esterolytic activities. Ongoing work is aimed at a better separation of the active proteins from this broad matrix and from one another. The checkered history and range of kinetic values in various published characterizations derive, at least in part, from inadequate separation and variations in purity of the crude extracts.

The commercially supplied product used in this investigation was prepared by the New Zealand Rennet Company Ltd. (Eltham, NZ). Their proprietary processing steps include extraction, concentration, filter-sterilization, and freeze-drying to yield a stable powdered product to which lactose is then added to give a standard TBG assay for commercial usage. Our starting material was the dried product without extender, thus permitting deletion of prepurification dialysis. This commercial product was further processed in our laboratory before being assayed against TOG (12) and TBG.

MATERIALS AND METHODS

Chemicals and Reagents

Freeze-dried pregastric lipase from lambs (New Zealand Rennet Company Ltd.) was the unextended product. The 4-NPA, TBG (96 to 98% grade II), 1,2-diacyl-*sn*-glycero-3-phosphocholine (L-α-lecithin), and reagent grade buffers, tris [tris(hydroxymethyl)aminomethane and 1,3 bis[tris(hydroxymethyl)-methylamino]propane) (**bis tris propane**)], (all from Sigma Chemical Co., St. Louis, MO) were used. Color key pH calibration buffers, laboratory grade 1,2-propanediol, and Folin and Ciocalteu phenol reagents were supplied by BDH Chemicals (Poole, UK). Sodium caseinate (Alanate 180) was supplied by the New Zealand Dairy Research Institute (Palmerston North, NZ). Fast flow Q-Sepharose was from Pharmacia LKB Biotechnology AB (Uppsala, Sweden). All water was Milli-Q grade, and resistance was typically >1.6 × 10^7 ohm/cm, processed with equipment from

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Millipore Corp (Bedford, MA). Instrument-grade dry nitrogen was supplied by New Zealand Industrial Gases Ltd. (Auckland, New Zealand).

Equipment

Solution pumping was carried out with a microtube pump (Eyela MP-3; Rikadenki Co., Tokyo, Japan). The column used for enzyme purification (Amicon-Wright, Beverly, MA) was of approximately 26-ml internal volume. Dialysis was carried out in 20DM dialysis tubing (Union Carbide, Chicago, IL). Spectral analyses were made on either an HP8452A diode array spectrophotometer controlled by an HP9000 series minicomputer (Hewlett Packard Co. Int., Palo Alto, CA) or a Varian Cary 219 spectrophotometer (Varian Associates Ltd., Palo Alto, CA). Both instruments were run with external water bath temperature controllers (Grant, Cambridge, UK). pH-stat titrations were carried out in temperature-jacketed vessels with a programmable autotitrator (Mettler DL21; Mettler Instruments AG, Greifensee, Switzerland). pH was measured with either a Mettler DG 111-SC (on the autotitrator) or an Ingold 104023365 combined pH microelectrode (Ingold Messtechnik AG, Switzerland) on a pH meter (Orion SA 520; Orion Research AG, Küsnacht, Switzerland). The ultrasonicator was an Elma T 460 (Hans Schmidtbauer KG, Germany). Mathematical data processing was predominantly carried out using Sigmaplot™ Version 5.0 (Jandel Scientific, Corte Madera, CA) on IBM-compatible personal computers. Contour plots were generated from the Sigmaplot™ interpolated data matrix using MathCad™ 4.0 for Windows (MathSoft Inc., Cambridge, MA).

Enzyme Processing

All work was carried out at approximately 4°C with degassed solutions, and dialysis was carried out under refrigeration. A column of fast-flow Sepharose-Q anion-exchange phase (ca. 16 mm i.d.; length = 130 mm) was preequilibrated with several volumes of 50 m*M* Tris·HCl buffer, pH 8.0. The crude raw product was suspended in 50 m*M* Tris·HCl buffer, pH 8.0, and allowed to equilibrate; after centrifugation, the supernatant was decanted and loaded onto the column. The column was first washed with the suspension buffer until no more protein was eluted; then, the total unbound material (fraction F1) was discarded. The column was then eluted with 50 m*M* acetate·HCl buffer, pH 4.6, to isolate a second fraction, F2, with high activity against 4-NPA but minimal activity against TBG. The final elution was carried out with 50 m*M* Tris·HCl at pH 8.0, containing 1 *M* NaCl to yield fraction F3, which was used in this study. The column phase was cleaned of residual protein with 0.5 *M* NaOH containing 1 *M* NaCl between loadings and stored in 20% (vol/vol) ethanol and water between runs.

Fraction F3 was dialyzed against several exchanges of 50 m*M* ammonium hydrogen carbonate to remove NaCl and the nonvolatile buffer. The dialyte was then frozen in liquid nitrogen and lyophilized to yield a slightly pink powder that was stored under instrument-grade dry nitrogen at –20°C until required for use.

Fresh stock was prepared by dissolving the powder in degassed Milli-Q water at 4°C. A 4-NPA assay (13) was used as an internal check for activity to monitor the change in absorbance at 400 nm wavelength for production of the 4-nitrophenolate ion from 1 m*M* 4-NPA in 50 m*M* bis tris propane, pH 7.2 at 37°C. Under these conditions, the activity of the lyophilized protein extract was 0.22 of absorbance min per mg (± <7%).

Protein Assay

The protein content was determined spectrophotometrically using the method of Lowry et al. (10) with bovine serum albumin as the standard. All determinations were made by interpolation onto a hyperbolic calibration curve (15, 16), fitted directly to the data for the protein standards by using the Marquardt-Levenberg fitting algorithm within Sigmaplot™ software. The determined protein content of the lipase preparation used in this study was 100% (± 5%), which does not mean that the protein was homogeneous.

TBG Assay

The following technique for the lipid assay provided the basis for the kinetic characterization across a range of pH and temperatures. The method is based upon the recommended procedure for the estimation of lipase and esterase activity from animal forestomach (2) and is very similar to the assay of Richardson and Nelson (18).

Because TBG is relatively insoluble in water, its exposure to the enzyme was as an emulsion. This presentation mimics that in nature: the lipase is active at the interface between aqueous and lipid phases during suckling and the early part of the digestive process. The two components of the emulsion-stabilizing base were prepared separately

before being mixed with the lipid and emulsified. A 10% (wt/vol) stock of L-α-lecithin in 1,2-propanediol was prepared by sonication. This stock could be stored at room temperature (13 to 25°C) indefinitely, but required redispersion immediately before mixing with the other component. Sodium caseinate (1.2 g) was dispersed in 100 ml of Milli-Q water by use of a magnetic stirrer, a further 100 ml of water were added, and the mixture again was stirred.

The two components in the emulsion base were mixed just prior to use. The lecithin-diol stock was resonicated in a bath of warm water (approximately 40°C), and 1 ml was pipetted with a warm pipette (to minimize aggregation and clogging) into the 200 ml of caseinate solution. This emulsifier preparation was then kept well stirred and provided multiple aliquots for use in the assay determinations of lipid hydrolysis.

The stock NaOH solution was prestandardized against oven-dried potassium hydrogen phthalate, and quality was maintained by careful filtration of CO_2 from the air vented into the holding flask.

A typical lipid emulsion stock was prepared by addition of 40 ml of the emulsifying base to TBG that had been weighed (17 to 22°C) in a dry, plastic titrator cup at room temperature. The cup and contents were then thoroughly sonicated in water bath at approximately 40°C. Dispersion was very good, and a standard emulsion character was established. [There is evidence that the final dispersed state of an emulsion of constant composition is primarily dependent on the power density in the ultrasonic bath (9).] Sonication at approximately 40°C also brought the emulsion mixture close to the temperature required for the titrimetric assay, thus reducing the time for final temperature equilibration with the water jacket used for temperature control.

The titrator cup and contents were then placed into the autotitrator, and stirring was maintained throughout the reaction. Final temperature and pH adjustments were made in situ. The pH was adjusted to the desired value using dilute HCl or dilute NaOH. Particular care was taken when the pH was adjusted to 5.5, which approaches the acid pH region at which precipitation of the caseinate component of the emulsion occurs. Too low a pH (or a microregion of low pH) quickly led to aggregation, potentially causing blockage of the electrode and sequestration of some of the substrate. If aggregation occurred, the preparation of the sample to be assayed was repeated.

At the beginning of an assay, an aqueous solution of the enzyme (ca. 5 mg/ml) was introduced by micropipette. A typical aliquot size was 100 μl but lay in the range 50 to 200 μl, depending on the activity of the enzyme under the reaction conditions.

An initial graphic output for the titration allowed for a rapid visual assessment of the stability and linearity of individual run data and identified a time window for rate calculations. A range of substrate concentrations was used for each specified condition of temperature and pH to provide data for full determinations of Michaelis-Menten substrate affinity constant ($\mathbf{K_m}$) and the apparent turnover number (K_{cat}). The use of K_{cat} is preferred to that of maximal velocity because the true concentration of pure enzyme is unknown, but the K_{cat} (rate per unit mass of our enzyme preparation) is well characterized (3).

The span of experimental conditions was from pH 5.5 to 7.5 inclusive, at unit intervals of 0.5 pH, and 30 to 50°C at intervals of 5°C. This range was sufficient to enclose the region of maximal activity. Polynomial interpolations, contours of constant activity, and the surface net values of K_{cat} for intermediate values at 1°C and unit intervals of 0.1 pH were generated, as detailed.

Mathematical Processing

The initial rate data for each Michaelis-Menten plot were fitted to two sorts of functions, a hyperbolic curve using the Marquardt-Levenberg algorithm within Sigmaplot®, and as a double reciprocal Lineweaver-Burk plot to which a linear least squares regression was fitted. For perfect data, the two procedures should produce the same kinetic parameters. For data with scatter, the Lineweaver-Burk plot differentially weights individual data points (7), leading to values for K_m and K_{cat} that differ from those obtained in the evenly weighted method of curve fitting. This difference, if any, was used to assess the quality of the fit, and the set of assays was repeated for any case exhibiting a difference >5%. The Michaelis-Menten parameters used herein were those derived directly from the hyperbolic least squares fit.

The total data file for K_{cat} was collated for processing into the interpolated final form of the kinetic surface plots. Third-order polynomial fits were used for precisely defined selections of constant pH and constant temperature, and then the surface net was constructed using the resultant polynomials. The original 25 K_m values (5 × 5 matrix) generated 441 values (21 × 21 matrix), giving a finer grain to the plot. This interpolation procedure was primarily introduced to suppress artifacts produced by the operation of the Sigmaplot® algorithim for surface net construction and has the secondary effect of slightly smoothing the data. This enlarged data file also

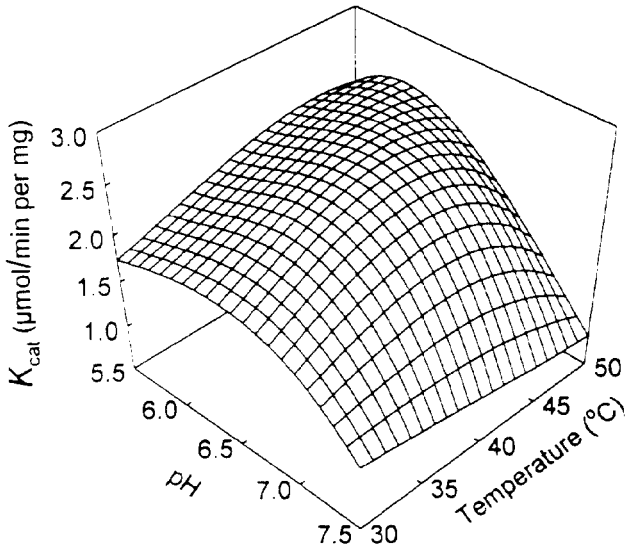

Figure 1. Kinetic surface plot of apparent turnover number (K_{cat}) against pH and temperature for the hydrolysis of tributyroylglycerol that was catalyzed by pregastric lipase of lambs.

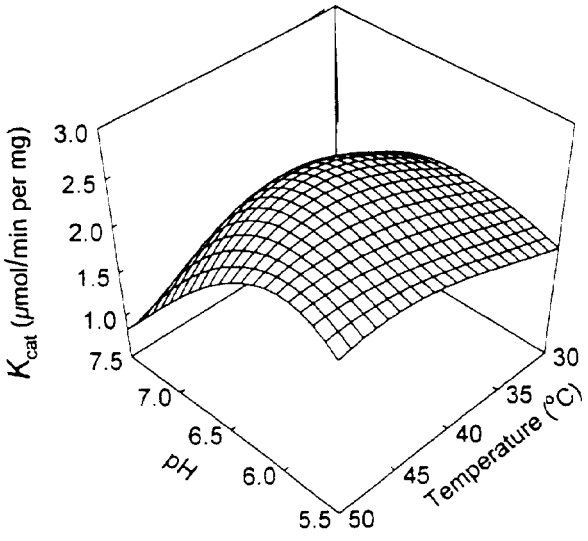

Figure 2. Obverse view of data for the kinetic surface plot of apparent turnover number (K_{cat}) against pH and temperature for the lamb hydrolysis of tributyroylglycerol that was catalyzed by pregastric lipase of lambs.

formed the basis for the MathCad™ calculation of the contour plot, and thus the contour plot exactly maps the surface net plots.

RESULTS AND DISCUSSION

The surface plot of K_{cat} for the experimental range of pH and temperature values is shown from two viewpoints in Figures 1 and 2. The point of maximum activity on the smoothed plot is at 43°C and pH 6.4, but a broad region of high activity surrounds this point that is best observed from the contour levels provided in Figure 3, which show activity lying within 10% of the maximum over the temperature and pH range of ca. ± 12°C and ± .6 pH units, respectively.

Experimentally, the activity was maximal at 40°C and pH 6.5. The K_m were generally low in this region, frequently < 0.2 m*M*, with some as low as 0.05 m*M*, indicating that the enzyme has a very high affinity for this substrate. The values for K_m showed significantly more scatter than K_{cat} in part because of the high substrate affinity (low K_m) and the consequent extreme sharpness of the Michaelis-Menten hyperbolas. At the very low substrate concentrations (below K_m values), good rate data were difficult to obtain, partly because of variable loss of substrate onto the walls of the vessel and other surfaces. Conversely, the K_{cat} obtained from the substrate saturation region (the extended and very flat plateau region of the Michaelis-Menten plots) were very reliable.

The maximal value of K_{cat} for F3 was ca. 2.6 μmol/min per mg compared with 0.7 μmol/min per mg obtained at pH 6.6 and 37.5°C for the unprocessed commercial product (13); K_m values of ≤ 0.2 m*M* were similar. The corresponding values of K_{cat} and K_m for activity of the identically processed extract against the lipid containing long-chain fatty acids, TOG, were 0.073 μmol/min per mg and 9 m*M*, respectively; pH 6.3 and 32°C were the optimal conditions (12).

Although milk fats do not contain TBG, butyric acid constitutes approximately 11% of the total fatty acids present within the constituent lipids and nearly 90% of this acid is at *sn*-3 position (17). The stereoselectivity of lipases for hydolyzing acyl residues in the *sn*-1,3 positions (8), particularly *sn*-3 for pregastric lipases (11, 14), is well documented; thus, triacylglycerols containing butyric acid in the *sn*-3 position would probably be the most appropriate substrates for a physiological study. However, a TBG assay, which is used in food industries (2) for standardizing the commercial product, also is an excellent assay for the partially purified lipase. Moreover, we have found (data not shown) that the enzyme frees the first butyric acid from TBG approximately 30 times more rapidly than it frees the second. Therefore, the data for hydrolysis of TBG will give some indication of the characteristics of the enzyme for

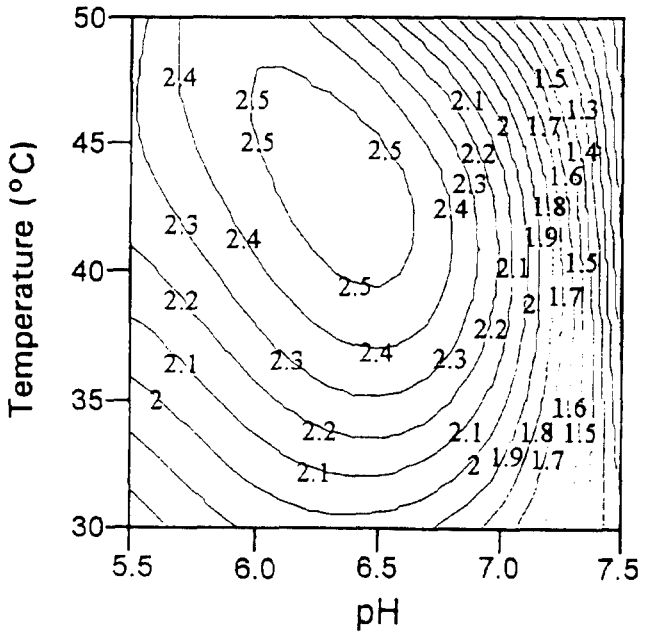

Figure 3. Contour plot of lines of constant apparent turnover number (K_{cat}) against pH and temperature for the lamb pregastric lipase catalyzed hydrolysis of tributyroylglycerol that was catalyzed by pregastric lipase of lambs.

mediating the production of butyric acid as a hydrolysate from milk fat, particularly when *sn* positional and chain length effects are resolved separately.

The 36:1 ratio of maximal turnover rates for TBG ($C_{12:0}$) over TOG ($C_{54:3}$) confirms the preference of the lipase for the lipid containing short-chain fatty acids, as do the K_m values for each substrate (45:1 ratio). Such preference by pregastric lipases is known from both relative rate data (4, 6, 11), and for the composition profile of the total free fatty acids released from bovine milk fat (1, 5). Preliminary work in this laboratory with $C_{18:0}$ to $C_{30:0}$ lipids clearly confirmed this preference. This short-chain dominated activity is important to the commercial role in milk fat processing for cheeses; for example, the Italian-style cheeses, such as Provolone and Romano, are largely characterized by the picante flavors of short-chain free fatty acids.

Modern techniques of biochemistry allow purification down to a single homogeneous protein if required, but, for a food-grade product, the processes are prohibitive. For this reason, the steps here are a compromise toward processing simplicity that offer some distinct advantages. In particular, the heme and polysaccharide fragments, as well as other unwanted components, are largely removed during centrifugation and selective binding, and the number of proteins present is reduced by the selective debinding. The cleaner background in the refined protein offers the advantages of reduced stray material and a cleanly soluble protein product, enhancing controlled dispersion. In subsequent production, substituting the use of dialysis with H_2O only has resulted in somewhat improved activity as well as a material that is even more readily soluble. This relatively simple process thus offers both enhanced specific activities and greater ease of controlled use.

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