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# Determining the regio- and typo-selectivity of calf pregastric lipase

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**Abstract**

A selection of natural lipids, milk fat, cocoa butter and soybean oil, and three synthetic triglycerides 1,3-dipalmitoyl-2-oleoyl-*rac*-glycerol (POP), 1(3),2-palmitoyl-3(1)-oleoyl-*rac*-glycerol (PPO), and 1,3-dipalmitoyl-2-butyryl-*rac*-glycerol (PBP) were used as substrates for calf pregastric lipase (CPGL) catalysed hydrolysis at pH 6.5, 37.5 °C. CPGL preferentially releases short-chain fatty acids at the *sn*-3 position and unsaturated fatty acids during the hydrolysis of lipids containing long-chain fatty acids, and its activity increases with increasing unsaturation in the carbon chain of the long-chain fatty acids. The relative rate constants of hydrolysis were 1.56, 0.99, 0.73 $h^{-1}$ for C4:0, C6:0, C8:0 esters, respectively, at *sn*-3; 0.0097, 0.0047 $h^{-1}$ for the C16:0, C18:0 esters at *sn*-1,3, respectively; 0.0094 $h^{-1}$ for the C18:1 ester at *sn*-2; and 0.0073, 0.0085, 0.012 $h^{-1}$ for C18:1, C18:2, C18:3 esters, respectively, at *sn*-1,2,3. These last results confirmed that CPGL enzyme does not exhibit positional selectivity towards lipid substrates. The rate constant of hydrolysis was 1.14 $h^{-1}$ for the C4:0 ester at *sn*-2.

PBP was synthesised in four steps from commercially available glycerol as starting material. Glycerol was first selectively protected as a bis-silylether at the primary alcohol (*sn*-1,3) positions. The free alcohol at the *sn*-2 position was then esterified with butyric anhydride. The protecting groups at C1 and C3 were then removed, and the resultant hydroxyl groups esterified with palmitic acid. 

*Keywords:* Calf pregastric lipase; 1,3-Dipalmitoyl-2-butyryl-*rac*-glycerol; Lipid hydrolysis; Selectivity; Triacylglycerols

## 1. Introduction

Dietary fats in mammals affect their health and ability to recover from disease. The assimilation of dietary fats into the body requires that they be pre-digested by pregastric lipases. Pregastric lipases are able to penetrate milk fat globules to catalyse the hydrolysis of the triacylglycerols therein. They are secreted from the oral glandular structures (lingual and pharyngeal tissues) of all mammals during suckling or swallowing [1].

All the ruminant pregastric lipases have selectivity for short-chain fatty acids. Their selectivity depends on the nature of the glycerides and the differences in their ability to release major *n*-chain fatty acids from various milk fats [2]. The lipases of calf, kid and lamb are typo-selective for hydrolysis of lipids with short-chain fatty acids [3] (typo-selectivity is the selectivity of a lipase for a lipid class or for the nature of the acyl chain). Both calf and lamb lingual lipases were more active against tributyrin than against the aryl ester, 4-nitrophenylacetate, or the longer chain lipid, triolein [4].

The calf, kid and lamb pregastric lipases all preferentially catalyse the hydrolysis of the *sn*-1 and *sn*-3 positions of the glycerides from ruminants' milk fat [2]. By utilising chiral and racemic triacylglycerols

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Table 1
Fatty acid composition and distribution in natural lipids, milk fat [8], cocoa butter [9] and soybean oil [9]

| Lipid | *sn* | Fatty acid | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 4:0 | 6:0 | 8:0 | 10:0 | 12:0 | 14:0 | 16:0 | 18:0 | 18:1 | 18:2 | 18:3 |
| Milk fat | *sn*-1 | 0.0 | 2.7 | 8.8 | 21.9 | 30.6 | 49.1 | 61.0 | 38.2 | | | |
| | *sn*-2 | 0.7 | 38.7 | 49.1 | 52.3 | 57.6 | 43.7 | 12.1 | 17.0 | | | |
| | *sn*-3 | 99.3 | 58.7 | 42.1 | 25.8 | 11.8 | 7.3 | 26.9 | 44.8 | | | |
| Cocoa butter | *sn*-1 | | | | | | | 47.1 | 47.9 | 11.4 | 12.6 | |
| | *sn*-2 | | | | | | | 2.4 | 2.0 | 80.7 | 83.5 | |
| | *sn*-3 | | | | | | | 50.6 | 50.1 | 7.9 | 3.8 | |
| Soybean oil | *sn*-1 | | | | | | | 49.6 | 50.0 | 31.6 | 29.6 | 37.0 |
| | *sn*-2 | | | | | | | 3.2 | 2.5 | 29.7 | 42.7 | 28.9 |
| | *sn*-3 | | | | | | | 47.1 | 47.5 | 38.7 | 27.7 | 34.2 |

differing in their fatty acid chain lengths, the three ruminant pregastric lipases were found to have *sn*-3 stereo-selectivity. The kid lipase was the most typo-selective and that from calf the least [3]. Calf pregastric lipase (CPGL) prefers substrates with short- and medium-chain fatty acid esters [5,6], and was found to exhibit very low activity against triacylglycerols composed of acyl groups of chain length greater than 16 carbons [7].

The short-chain fatty acids in milk fat are predominantly at the *sn*-3 position (Table 1). Although milk fat has a complex composition containing many fatty acids, the rate of release of the eight most abundant fatty acids from milk fat, catalysed by CPGL, has been used in this study to compare the effect of chain length and positional selectivity of the enzyme for catalysing the hydrolysis of a natural fat. Natural fats are routinely used in industry to evaluate lipase activity.

Cocoa butter and soybean oil, which both contain a majority of long-chain and unsaturated fatty acids (Table 1) were selected as substrates for evaluation of the preference of CPGL for unsaturated fatty acids. Cocoa butter was chosen in preference to a single synthetic long-chain lipid because it is a natural lipid widely used in the food industry. Cocoa butter contains about 45% of a racemic triglyceride, 1(3)-palmitoyl-2-oleoyl-3(1)-stearoyl-*rac*-glycerol (POS), and the total proportion of triglycerides containing palmitic and stearic acid located in the *sn*-1,3 positions and oleic acid in the *sn*-2 position is typically over 80% (Table 1).

Soybean oil contains significant levels of stearic, oleic, linoleic and linolenic acid. The positional fatty acid distribution of soybean oil (Table 1) is more uniform than that of cocoa butter, particularly for C18:1, C18:2 and C18:3, and so it was chosen as the substrate for further evaluation of the unsaturated fatty acid preference of CPGL. Stereo-selectivity was further confirmed by evaluating rate constants for CPGL catalysed hydrolysis of the esters present in 1,3-dipalmitoyl-2-butyryl-*rac*-glycerol (PBP), 1,3-dipalmitoyl-2-oleoyl-*rac*-glycerol (POP) and 1(3),2-palmitoyl-3(1)-oleoyl-*rac*-glycerol (PPO).

## 2. Materials and methods

PBP was synthesised as described below. POP, PPO valeric acid (5:0) (>98%), tridecanoic acid (13:0) (99%), *tert*-butyldimethylsilyl chloride, imidazole, tributyrylglycerol (TBG) (96–98% grade II) were from Sigma.

Milk fat was provided by the New Zealand Dairy Board. Cocoa butter was obtained from a local confectionery maker and soybean oil was supplied from AMCO Produce Inc. Sodium dihydrogen phosphate ($NaH_2PO_4{\cdot}2H_2O$) was from Fluka-Garantie. Hydrochloric acid (AR), diethyl ether (AR), hexane (HPLC), disodium hydrogen orthophosphate anhydrous ($Na_2HPO_4$), and *iso*-octylphenoxypolyethoxyethanol (Triton X-100), palmitic acid, butyric anhydride, 4-(dimethylamino) pyridine (DMAP) and dicyclohexylcarbodiimide (DCC) were supplied by BDH, Amberlyst$^{TM}$ A-26 ion exchange resin was supplied by Acros, glycerol by Ajax Chemicals and tetrabutylammonium fluoride (TBAF) by Riedel-de-Haën.

Synthetic reactions were carried out under an atmosphere of dry nitrogen. All chemicals were reagent grade and used without further purification, except for some solvents. Tetrahydrofuran (THF) and diethyl ether were freshly distilled from sodium/benzophenone. Dichloromethane ($CH_2Cl_2$) was freshly distilled from calcium hydride. Pyridine and triethylamine ($Et_3N$) were dried and distilled from calcium hydride and stored over potassium hydroxide. Dimethylformamide (DMF) was dried and vacuum distilled from calcium hydride and stored over molecular sieves. Reactions were followed by TLC on Merck 0.25 mm silica gel on aluminium backed sheets. The $R_f$ values refer to TLC on silica gel. NMR spectra were recorded on a Bruker DRX-400 or a Bruker AM-200 spectrometer operating at 400 MHz ($^1$H)/100 MHz ($^{13}$C) or 200 MHz ($^1$H)/50 MHz ($^{13}$C), respectively. Proton NMR data are reported in ppm downfield from tetramethylsilane as internal standard. Carbon NMR data are reported in ppm relative to solvent as internal standard. Mass spectra were recorded on a VG 7070 mass spectrometer, operating at a nominal accelerating voltage of 70 eV. Infrared (IR) spectra were obtained as a sodium chloride disc using a Perkin-Elmer Paragon 1000PC.

### 2.1. Enzyme

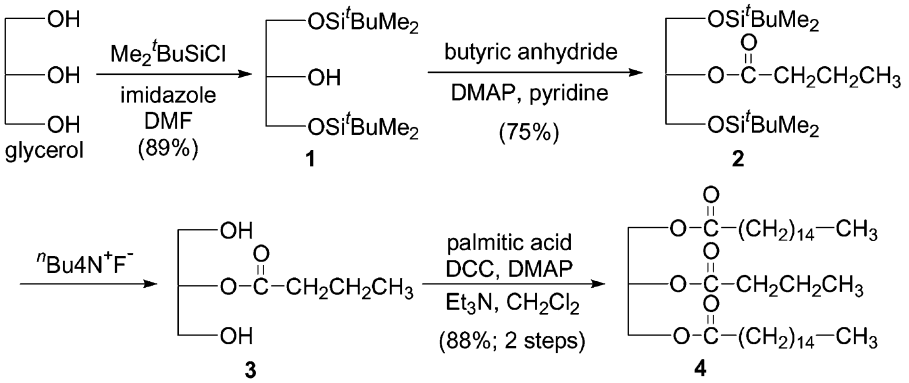
Freeze–dried pregastric lipase from calves (CPGL) was supplied by the New Zealand Rennet Co., Ltd. The partial purification of CPGL was performed as described previously [5]. The enzyme was shown to have lipase activity of 6.30 μmol $min^{-1}$ $mg^{-1}$ of enzyme against TBG (8.3 mM), at 37 °C and pH 6.5. The purification factor was 3.31.

### 2.2. Synthesis of 1,3-dipalmitoyl-2-butanoyl-rac-glycerol (4)

1,3-Dipalmitoyl-2-butanoyl-*rac*-glycerol (**4**) was synthesised as a lipase substrate with a butyl group located at the *sn*-2 position. The synthesis of the triacylglycerol (**4**) began with commercially available glycerol (Scheme 1) which was protected at C-1 and C-3 as a *tert*-butyldimethylsilyl ether. Bis-silyl ether (**1**) was then esterified with butyric anhydride to afford a butyrate ester at C-2. The silyl protecting groups were then removed, and the unprotected hydroxy groups at C-1 and C-3 in diol (**3**) were converted to palmitoyl esters to give the desired compound **4**. The synthetic route adopted is summarised in Scheme 1.

#### 2.2.1. 1,3-Bis[(1,1-dimethyl)ethyldimethylsilyloxy]-2-propanol (1)

A solution of glycerol (2.04 g, 22.1 mmol) in DMF (14 ml) was stirred at 0 °C for 3 min. A solution of *tert*-butyldimethylsilyl chloride (6.7 g, 44.2 mmol) and imidazole (3.3 g, 44.2 mmol) in DMF (12 ml) was slowly added to the reaction mixture at 0 °C under an atmosphere of nitrogen and the resultant mixture was warmed to ambient temperature overnight. Water (30 ml) was added and the aqueous layer was extracted with diethyl ether (3 ml × 30 ml). The




Scheme 1. Synthesis of 1,3-dipalmitoyl-2-butanoyl-*rac*-glycerol (**4**).

combined organic extracts were washed with saturated ammonium chloride solution (4 ml × 20 ml), dried over $MgSO_4$, filtered, and concentrated in vacuo to give 6.4 g (90%) of the crude bis-silyl ether. The crude viscous oil was purified by flash chromatography (eluting with ethyl acetate–hexane = 1:5) to give 1,3-bis[(1,1-dimethyl)ethyldimethylsilyloxy]-2-propanol (**1**) (5.99 g, 85%) as a colourless oil. The $R_f$ (5:1 hexane –ethyl acetate): 0.43; $^1$H NMR (200 MHz, $CDCl_3$): $\delta$ 0.07 (12H, s, 2× $Me_2Si$), 0.90 (18H, s, 2× *t*-Bu), 2.46 (1H, br. s, OH), 3.64 (5H, s, 2× $CH_2O$ and CHO); $^{13}$C NMR (50 MHz, $CDCl_3$): $\delta$ −5.4 ($CH_3$, $Me_2Si$), 18.3 (C, *t*-Bu), 25.9 ($CH_3$, *t*-Bu), 63.5 ($CH_2$, $CH_2O$), 71.9 (CH, CHO); HRMS calcd. for $C_{15}H_{37}Si_2O_3$ ($MH^+$): 321.22752; observed: 321.22813.

### 2.2.2. 1,3-Bis[(1,1-dimethyl)ethyldimethylsilyloxy] prop-2-yl butanoate (**2**)

To a stirred solution of bis-silyl ether (**1**) (5.99 g, 18.5 mmol) in dry pyridine (60 ml), was added DMAP (0.23 g) and butyric anhydride (20 ml) dropwise at 0 °C. The mixture was warmed to ambient temperature under an atmosphere of nitrogen over 1 h, then heated under reflux on a sand bath overnight. The reaction mixture was quenched by slow addition of water (30 ml) and the resultant mixture was slowly poured (over 30 min) into saturated $NaHCO_3$ (100 ml) with rapid stirring at 0 °C until no further effervescence occurred. The resultant mixture was then extracted with ethyl acetate (3 ml × 100 ml). The combined organic extracts were washed with ice-cold dilute HCl (1 M, 50 ml), water (50 ml) and saturated NaCl (50 ml). After drying over $MgSO_4$, filtration and concentration in vacuo afforded the desired product as a pale yellow viscous oil which was further purified by flash chromatography (eluting with ethyl acetate–hexane = 1:5) and concentrated in vacuo to give 1,3-bis[(1,1-dimethyl)ethyldimethylsilyloxy] prop-2-yl butanoate (**2**) (5.5 g, 75%) as a colourless oil. The $R_f$ (5:1 hexane–ethyl acetate) 0.62; $^1$H NMR (200 MHz, $CDCl_3$): $\delta$ 0.04 (12H, s, 2× $SiMe_2$), 0.87 (18H, s, 2× *t*-Bu), 0.94 (3H, t, $J$ = 6.5 Hz, $CH_3$), 1.60–1.71 (2H, m, $CH_2$), 2.30 (2H, t, $J$ = 6.59 and 6.99 Hz, $CH_2$), 3.69 (4H, d, $J$ = 5.32 Hz, $CH_2O$), 4.01–4.32 (1H, m, CHO); $^{13}$C NMR (50 MHz, $CDCl_3$): $\delta$ −5.5 ($CH_3$, $Me_2Si$), 13.6 ($CH_3$, butyl ester), 18.2 (C, *t*-Bu), 18.4 ($CH_2$, butyl ester), 25.7 ($CH_3$, *t*-Bu), 36.2 ($CH_2$, butyl ester), 61.4 ($CH_2$, $CH_2O$), 71.7 (CH, CHO), 172.9 (C, C=O); HRMS calcd. for $C_{19}H_{43}Si_2O_4$ ($MH^+$): 391.26999; observed: 391.26968.

### 2.2.3. 1,3-Dihydroxyprop-2-yl butanoate (**3**)

To a solution of bis-silyl ether (**2**) (5.6 g, 14.1 mmol) in dry THF (100 ml) at room temperature under an atmosphere of nitrogen, was added tetrabutylammonium fluoride (35.2 ml of a 1.0 mol l$^{-1}$ solution in THF, 35.2 mmol). The reaction mixture was stirred for 2 h then concentrated in vacuo. The resultant residue was purified by flash chromatography (eluting with ethyl acetate–hexane = 4:1) to give 1,3-dihydroxyprop-2-yl butanoate (**3**) (1.9 g, 78%) as a colourless oil which was used directly in the next reaction. The $R_f$ (1:4 hexane–ethyl acetate): 0.53; $^1$H NMR (200 MHz, $CDCl_3$): $\delta$ 0.96 (3H, t, $J$ = 7.34 and 7.43 Hz, $CH_3$), 1.61–1.73 (2H, m, $CH_2$), 2.34 (2H, t, $J$ = 7.53 Hz, $CH_2$), 3.68 (4H, d, $J$ = 3.78 Hz, $CH_2O$), 4.15–4.19 (1H, m, CHO).

### 2.2.4. 1,3-Dipalmitoyl-2-butanoyl-rac-glycerol (PBP) (**4**) [10]

To a solution of palmitic acid (6.2 g, 24.1 mmol) and DCC (4.98 g, 24.1 mmol) in dry $CH_2Cl_2$ (50 ml) was added butanoate **3** (1.7 g, 10.4 mmol), DMAP (0.26 g, 2.1 mmol) and $Et_3N$ (4.5 ml, 32.4 mmol) at 0 °C under nitrogen. The resultant mixture was stirred for 5 min at 0 °C then at 20 °C for 3 h. The urea that precipitated was removed by filtration and the filtrate was concentrated in vacuo. The resultant residue was dissolved in $CH_2Cl_2$ and the solution washed with 0.5 M HCl (2 ml × 25 ml), saturated $NaHCO_3$ (50 ml), dried over $MgSO_4$, then filtered and concentrated in vacuo. The resultant crystalline product was purified by flash chromatography (eluting with ethyl acetate–hexane = 1:50) to give 1,3-dipalmitoyl-2-butanoyl-*rac*-glycerol (**4**) (5.9 g, 88%) as a colourless lipid. The $R_f$ (5:1 hexane–ethyl acetate): 0.60; $8_{max}$ 1737.07 (C=O); $^1$H NMR (400 MHz, $CDCl_3$): $\delta$ 0.86–0.92 (6H, m, $CH_3$ from palmitic ester), 0.94 (3H, t, $J$ = 7.4 Hz, $CH_3$ of butyl ester), 1.26–1.28 (56H, m, $CH_2$ of palmitate), 1.64 (2H, t, $J$ = 7.4 Hz, $CH_2$ of butyl ester), 2.28–2.33 (2H, m, $CH_2$ of butyl ester), 4.30 (4H, d, $J$ = 4.2 Hz, $CH_2O$), 5.25–5.28 (1H, m, CHO); $^{13}$C NMR (100 MHz, $CDCl_3$): $\delta$ 13.6 ($CH_3$, but C1, 1′), 14.1 ($CH_3$, but C22), 18.3 ($CH_2$, but C21), 22.7 ($CH_2$,

pal C2, 2′), 24.8 ($CH_2$, pal C3, 3′), 24.9 ($CH_2$, pal C4, 4′), 29.0 ($CH_2$, pal C5, 5′), 29.1 ($CH_2$, pal C6, 6′), 29.2 ($CH_2$, pal C7, 7′), 29.3 ($CH_2$, pal C8, 8′), 29.4 ($CH_2$, pal C9, 9′), 29.6 ($CH_2$, pal C10, 10′), 29.7 ($CH_2$, pal C11, 11′), 31.9 ($CH_2$, pal C12, 12′), 34.0 ($CH_2$, pal C13, 13′), 34.2 ($CH_2$, pal C14, 14′), 35.9 ($CH_2$, pal C15, 15′) 36.2 ($CH_2$, but C20), 62.0 ($CH_2$, $CH_2O$), 68.8 (CH, CHO), 172.8 (C, C=O of but), 173.0 (C, C=O of pal); HRMS calcd. for $C_{39}H_{74}O_6$ ($M^+$): 638.54854; observed: 638.54758.

### 2.3. CPGL catalysed hydrolysis of natural fats and synthetic triglycerides

Hydrolysis reactions of milk fat, cocoa butter and soybean oil were carried out in an emulsion or oil prepared by stirring and ultrasonication of a mixture of molten lipid, Triton X-100 (75 mg) and buffer ($NaH_2PO_4.2H_2O/Na_2HPO_4$ 0.2 M, pH 6.5, 8 ml) for 30 min. After this time, this emulsion was placed in a water bath (37.5 °C) to stabilise the reaction conditions and 400 μl of CPGL (17.5 mg $ml^{-1}$) was added with an Eppendorf pipette. Samples (1 ml) were taken for analysis of hydrolysis of milk fat after 0, 15, 30, 45, 60, 90 and 120 min and of cocoa butter/soybean oil at 0, 1, 2, 4, 6, 8, 10, and 24 h. The reaction was quenched by immediate addition of the sample into a solution of acidified diethyl ether. Further extraction was then possible.

The free fatty acid (FFA) composition and triglyceride fatty acid (FA) composition were analysed by gas chromatography (GC).

In the experiments designed to study, the hydrolysis of synthetic triglycerides, 100 mg of POP or PBP were added to buffered emulsion (4 ml), prepared as described above. In the case of PPO, 20 mg were added to 1 ml of buffered emulsion. After sonication of the emulsion, 200 μl of freeze–dried CPGL (17.5 mg $ml^{-1}$) was added to 4 ml emulsion (50 μl to 1 ml PPO emulsion), held at 37.5 °C, to initiate the hydrolysis reaction. Samples (800 μl) were removed for analysis after different times adjusted to accommodate the rate of hydrolysis of each substrate (15, 30, 60 and 120 min for PBP; 1, 2, 3 and 6 h for POP; 2 h for PPO). The amount of FFA released by hydrolysis of the synthetic triglycerides was analysed by GC.

### 2.4. Preparation of triglyceride FAs and FFAs for analysis by GC

Extraction of lipids was carried out in a 40 ml Teflon coated screw-top centrifuge tube, which was kept in an icebox throughout the procedure. An ice-cold mixture of diethyl ether (20 ml) and HCl (3 ml, 35% v/v aqueous HCl) was added into a screw-top centrifuge tube. Internal standards (0.5 ml, mixture of valeric acid (1 mg $ml^{-1}$) and tridecanoic acid (1 mg $ml^{-1}$) in hexane) were added. The sample (1 ml), prepared as Section 2.3, was added into the mixture and Milli-Q water (5 ml) was added. The centrifuge tube was sealed and shaken for 3 min on a Vortex mixer, then centrifuged at 2330 × $g$ for 10 min. The top layer of the centrifuged sample (7 ml) was removed by Pasteur pipette and dried over anhydrous $Na_2SO_4$ (450 mg) for 20 min. The sample was then ready to be removed for further treatment.

Amberlyst™ A-26 ion exchange resin was washed ready for use. The resin (20 g) was shaken for 15 min with NaOH solution (20 ml, 1 M) and then with Milli-Q water (300 ml) three times, with a 10 min shaking between each wash. Finally, the resin was washed with methanol (150 ml) three times, again with 10 min shaking between each wash, and stored in methanol to be used for separation of FFA from unhydrolysed lipids. The dried ether-extract (4 ml) was added to methanol (2 ml) and wet resin (200 mg) in a 10 ml tall test tube with a plastic lid. The well-sealed test tube was shaken horizontally on a mechanical shaker at 120 shakes $min^{-1}$ for 1 h. The solvent was removed and the resin washed five times with an ether/methanol mixture (6 ml, 2:1 v/v).

Acetyl chloride (3 ml) was added dropwise to ice-cold methanol (10 ml) to prepare methylating reagent (5% v/v HCl in methanol) according to the method used by Carroll [11]. The methylating reagent (1 ml) was added to the separated FFA and the test tube was placed in the water bath at 40 °C overnight. Hexane (0.5 ml) was added to the dried ether-extract (1 ml) followed by methylating reagent (1 ml) in a short test tube with a glass lid. The well-sealed test tube was also left in the water bath at 40 °C overnight. During methylation, the test tubes for FFA and total FA were well-sealed to avoid the escape of volatile short-chain fatty acid methyl esters. The next day, all the test tubes were removed from the water bath and

cooled before addition of hexane (1 ml). The mixture was gently shaken on a Vortex mixer for 2 min, and then saturated NaCl (1 ml) was added, before shaking again for 20 s. The hexane layer was removed and placed in a GC vial.

### 2.5. Analysis of fatty acids by gas chromatography

GC analysis of fatty acid methyl esters (FAMEs) was performed on a Hewlett Packard 5890 series II Gas Chromatograph using a J & W DB-225 column (0.25 μm film thickness, 15 m length, 0.243 mm internal diameter). A flame ionisation detector was used at 250 °C. The carrier gas was helium and the column head pressure was set to 10 psi. Each sample (1 μl) of fatty acids was injected using a HP7673 automatic sampler using a split injection system at a temperature of 250 °C. The oven temperature was held at 40 °C for 3 min after injection of the sample. The temperature was then increased by 8 °C $min^{-1}$ to a final setting of 220 °C and held at this temperature for 1 min. Results from each injection were integrated automatically using the HP GC Chemstation program (Hewlett Packard) run on a Pentium III computer. Peaks were identified by using FAME standards prepared from FFAs using the methylation procedure.

## 3. Results

### 3.1. CPGL catalysed hydrolysis of milk fat

The extent of hydrolysis of milk fat, catalysed by CPGL in a Triton X-100 emulsion at pH 6.5 and 37.5 °C, over various periods of time, calculated as a percent of total FFA compared with the total content of fatty acid in the milk fat, increased from 4.7% after 15 min to 46.7% after 120 min. The %hydrolysis of each fatty acid ester was calculated as the percent of free acid compared with the total content of fatty acid present in milk fat (Fig. 1) and the rates of hydrolysis of each short-chain fatty acid ester (C4:0, C6:0 and C8:0) were fitted well on linear plots of ln(100 − %hydrolysis) against time (Fig. 2), thus, leading to evaluation of rate constants for hydrolysis.

### 3.2. CPGL catalysed hydrolysis of cocoa butter and soybean oil

The extent of hydrolysis of cocoa butter catalysed by CPGL in a Triton X-100 emulsion at pH 6.5 and 37.5 °C, over various periods of time, which was calculated as a percent of total FFA compared with the total content of fatty acid in the cocoa butter, increased from 1.5% after 1 h to 11.3% after 24 h. The extent of hydrolysis of soybean oil, calculated as a percent of

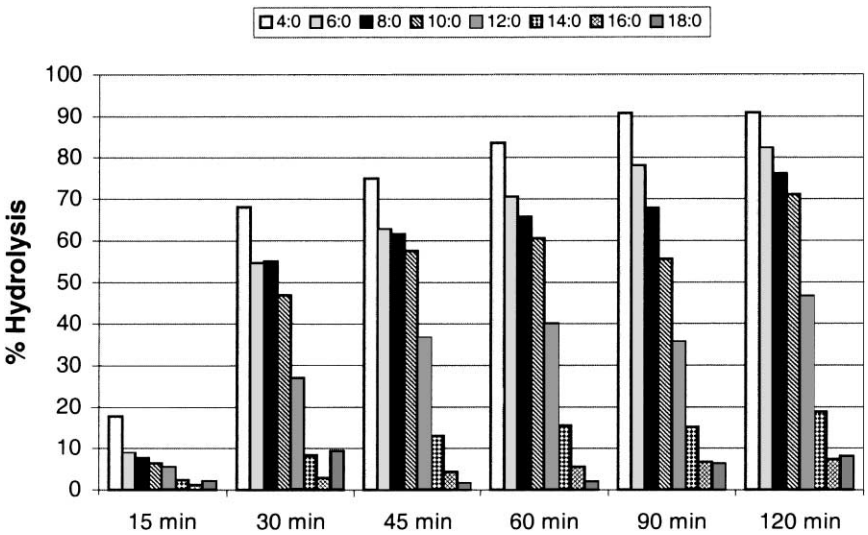



Fig. 1. The %hydrolysis of fatty acid esters in milk fat calculated as the percent of free acid compared with the total content of fatty acid.

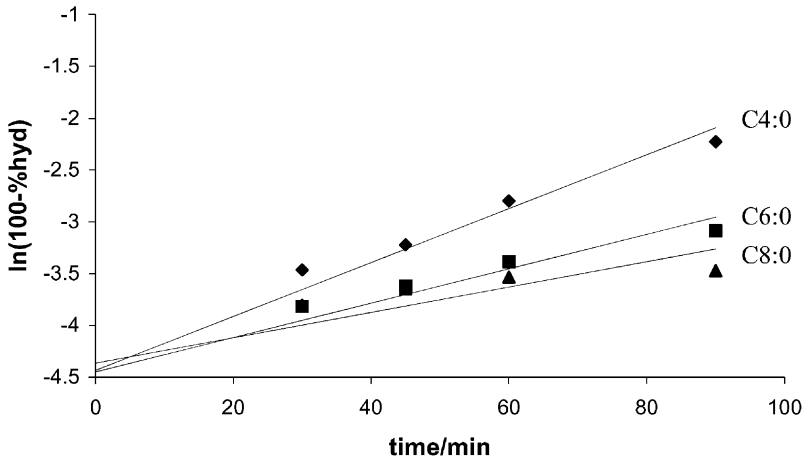



Fig. 2. Time dependence of the rate of hydrolysis of fatty acid esters (C4:0, C6:0 and C8:0) present in milk fat.

total FFA compared with the total content of fatty acid in the soybean oil, increased from 1.7% after 1 h to 5.8% after 24 h. The rates of hydrolysis of each fatty acid ester in both substrates were fitted well on linear plots of ln(100 − %hydrolysis) against time, (Fig. 3 for cocoa butter, Fig. 4 for soybean oil), thus, allowing the relative rate constants of hydrolysis to be evaluated.

### *3.3. CPGL catalysed hydrolysis of synthetic triglycerides*

The %hydrolysis of each FFA released from POP, compared with the total fatty acids present, showed 9.2% release of C16:0 and 3.1% release of C18:1 after 6 h hydrolysis. The rates of hydrolysis of each fatty acid ester were fitted well on linear plots of ln(100 − %hydrolysis) against time (data not shown).

The mol% of each FFA released from PPO after 2 h catalysed hydrolysis was compared with the total content of free fatty acids present in PPO and with the mol% of each FFA released from POP after the same time period of catalysed hydrolysis. The relative mol% data for C16:0/C18:0 are control (unhydrolysed PPO): 69.0/31.0; PPO: 76.2/23.8; POP: 79.8/20.2, respectively. It was not possible to evaluate the rate

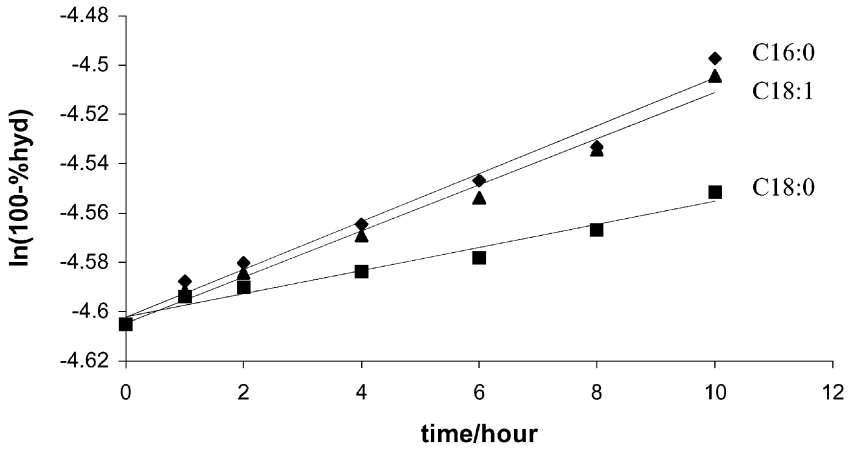



Fig. 3. Time dependence of the rate of hydrolysis of fatty acid esters (C16:0, C18:0 and C18:1) present in cocoa butter.

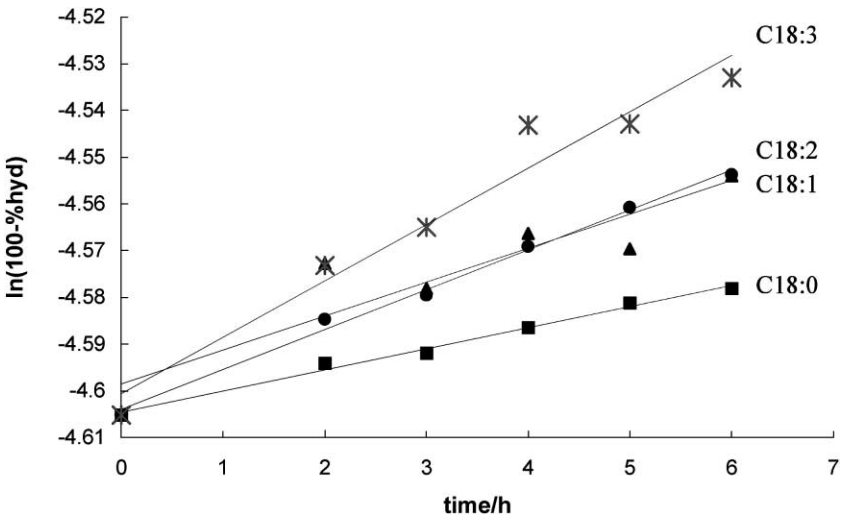



Fig. 4. Time dependence of the rate of hydrolysis of fatty acid esters (C18:0, C18:1, C18:2 and C18:3) present in soybean oil.

constants of each fatty acid in PPO from the limited data available.

The %hydrolysis of each fatty acid, calculated as the percent of free acid compared with the total content of fatty acid present in PBP, showed 88.6% release of C4:0 and 31.2% release of C16:0 after 120 min hydrolysis. The mol% of each FFA released from PBP after 2 h was compared with the total content of free fatty acids. The relative mol% data for C4:0/C16:0 are control: 28.3/71.7; after 2 h: 56.6/43.4. The rates of hydrolysis of each fatty acid ester were fitted well on linear plots of ln(100 − %hydrolysis) against time (Fig. 5), thus, allowing evaluation of the relative rate constants of hydrolysis.

The values of the rate constants of CPGL catalysed hydrolysis of the fatty acid lipid esters, obtained from the natural fats and synthetic triglycerides used in this investigation are summarised in Table 2.

### 3.4. Synthesis of the PBP triacylglycerol

In the synthesis of the PBP triacylglycerol the step where acyl migration may have occurred was in the desilyation of bis-silyl ether **2** to diol **3**. The $^{1}$H and

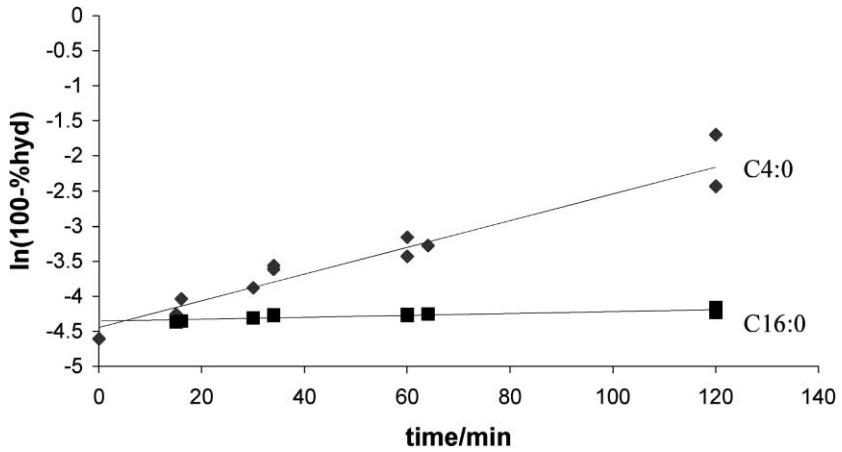



Fig. 5. Time dependence of the rate of hydrolysis of each fatty acid ester (C4:0 and C16:0) present in PBP.

Table 2
The rate constants of CPGL catalysed hydrolysis of several fatty acid lipid–esters located at different positions on the glycerol backbone

| Ester | *sn*-1,3 ($h^{-1}$) | *sn*-2 ($h^{-1}$) | *sn*-3 ($h^{-1}$) | *sn*-1,2,3 ($h^{-1}$) |
|---|---|---|---|---|
| C4:0 | | 1.14 | 1.56 | |
| C6:0 | | | 0.99 | |
| C8:0 | | | 0.73 | |
| C16:0 | 0.0097 | | | |
| C18:0 | 0.0047 | | | |
| C18:1 | | 0.0109 | | 0.0073 |
| C18:2 | | | | 0.0085 |
| C18:3 | | | | 0.012 |

$^{13}$C NMR spectrum of **3** provides clear evidence that acyl migration did not occur in that there is only a methine proton at C-2 in the $^{1}$H NMR spectrum (and the corresponding carbon in the $^{13}$C NMR spectrum) is de-shielded which is consistent with the C-2 position being acylated. Acylation at C-1 and/or C-3 would necessitate that a two proton methylene group be de-shielded which is clearly not the case.

## 4. Discussion

### *4.1. Hydrolysis of natural lipids catalysed by CPGL*

The selectivity of a lipase controls the extent of hydrolysis of triglycerides and it has been well established that CPGL has a strong preference for catalysing the hydrolysis of the short-chain fatty acid esters [4–6]. Milk fat has 16.3% of short-chain fatty acids (C4–C8), and most of them occupy the *sn*-3 position (Table 1). The released fatty acid profile at various stages of hydrolysis of milk fat, catalysed by CPGL, shows that 84% of butyric acid (C4:0) is released after 1 h hydrolysis (Fig. 1). The %hydrolysis decreased to 71% for caproic acid (C6:0), and further decreased to 66% for C8:0, 61% for C10:0, and 40% for C12 (after 1 h hydrolysis). For the long-chain saturated fatty acids (C14:0, C16:0 and C18:0), the %hydrolyses were only 15, 6, and 2%, respectively, (after 1 h hydrolysis). Moreover, the hydrolysis of milk fat catalysed by CPGL commenced very rapidly (36% hydrolysis was achieved in 1 h), but then the reaction slowed down to a further 10% hydrolysis over the next 1 h. These observations can be fully explained by the selectivity properties of CPGL and preferential release of short-chain fatty acids. However, hydrolysis also seems to have occurred from both the *sn*-1 and *sn*-2 positions, as evidenced by the significant amount of hydrolysis of the longer chain fatty acid ester, e.g. C14:0 which shows 19% hydrolysis after 120 min when it is primarily located at the *sn*-1 and *sn*-2 positions. Therefore, in order to detect positional selectivity, a substrate with a restricted number of fatty acids (cocoa butter) was chosen for continuation of this study.

It is seen in Fig. 3 that the activity of CPGL against the fatty acid esters in cocoa butter increases with increasing unsaturation in the C18 carbon chain, i.e. the rate of catalysed hydrolysis of the lipid–ester with C18:1 is greater than that for C18:0. Moreover, as for the short-chain fatty acid esters, increasing chain length of fatty acid esters decreases the rate of hydrolysis, with the C18:0 ester being hydrolysed more slowly than the C16:0 ester.

Cocoa butter is a mixture of triglycerides with relatively long-chain fatty acid chains, and a distribution of unsaturated fatty acids. After 24 h hydrolysis, the released fatty acid profile showed 27% linoleic acid (C18:2, the most unsaturated fatty acid). However, the %hydrolysis decreased to 13% oleic acid (C18:1), and further decreased to 7% stearic acid (C18:0) after 24 h hydrolysis. These results show that CPGL has a preference for releasing unsaturated fatty acids, a result which was not detectable in tests on the selectivity of the pregastric lipases from lamb and goat [12]. Moreover, the results suggest that the location of the fatty acid does not necessarily deter its subsequent lipolysis. Normally, lipases exhibit positional, or regio-selectivity wherein they preferentially catalyse the hydrolysis of ester bonds either at the primary, or more rarely, the secondary position.

In contrast to milk fat, the oleic acid (C18:1) content in cocoa butter is typically located (>80%) in the *sn*-2 position. The preference for oleic acid (C18:1) hydrolysis compared with stearic acid (C18:0), which is almost equally located at the *sn*-1,3 positions, might therefore, have been attributed to non-positional selectivity (or indeed *sn*-2 selectivity) rather than preference for release of an unsaturated fatty acid. It was, therefore, desirable to use another natural fat, soybean oil, with a more even positional distribution of fatty acids.

CPGL hydrolyses palmitic acid (C16:0) (data not shown) and oleic acid (C18:1) in soybean oil at similar rates, with the hydrolysis of stearic acid (C18:0) being much slower. Both palmitic and stearic acids are evenly distributed over the *sn*-1,3 positions, and therefore, direct comparison of rate data is valid. Oleic acid, however, has almost one-third of its substitution at *sn*-2, and this is likely to contribute to the overall rate of hydrolysis. Nevertheless, the trends observed are similar to those observed for CPGL hydrolysis of cocoa butter catalysed by CPGL. The data confirm the preference of CPGL for catalysing the hydrolysis of unsaturated fatty acids, with a greater degree of unsaturation leading to more preferred hydrolysis. After 24 h hydrolysis of soybean oil, the profile of released fatty acids shows that 5% oleic acid (C18:1) was released, with an increase to 7% linoleic acid (C18:2) and a further increase to 11% linolenic acid (18:3). Each of these three acids is distributed over all three *sn*-1, *sn*-2 and *sn*-3 position, within a range of $\pm 10\%$.

In summary, the use of milk fat, cocoa butter and soybean oil as substrates for evaluating lipase selectivity has shown that CPGL has selectivity towards catalysed hydrolysis of short-chain fatty acid esters, but non-specific positional (regio-) selectivity for the primary (*sn*-1 and *sn*-3) and secondary (*sn*-2) ester bonds of a triglyceride. However, CPGL, unlike other pregastric lipases [12], has a strong preference for releasing unsaturated fatty acids.

Comparison of the selectivity of a lipase for various natural or synthetic lipids is relevant only for a minor extent of hydrolysis. For this reason, we have restricted quantitative comparison to data which complied with the following restrictions: overall extent of hydrolysis $<10\%$; extent of hydrolysis of every acyl chain $<10\%$. Thus, the data shown in Figs. 3 and 4 do not include results where the extent of hydrolysis is high and questionable. Indeed, curvature in the plots was seen when the data for 24 h hydrolysis were included. Non-linearity is likely to arise when acyl migration within the lipid increases to such an extent that positional specificity becomes scrambled.

It is acknowledged that allocation of the rate constants to specific locations on the triglycerides is made on the basis of major positional allocation (e.g. C18:1 in cocoa butter which is 87.4% at *sn*-2) or on average positional allocation (e.g. C18:1, C18:2 ad C18:3 are approximately evenly spread over the *sn*-1, *sn*-2 and *sn*-3 positions in soybean oil). Nevertheless, it now becomes clear that CPGL is not regio-selective, since catalysed hydrolysis of esters has taken place at all of the *sn*-1, *sn*-2 and *sn*-3 positions. The shorter the saturated carbon chain, the greater the degree of catalysis. The more unsaturated the acid the greater is the degree of hydrolysis. The difference in the two values of the rate constants for hydrolysis of the C18:1 ester ($0.0094\,h^{-1}$) at *sn*-2, $0.0073\,h^{-1}$ at *sn*-1,2,3 was too small to be able to reach a conclusion on the relative enzymic activity towards an ester located at *sn*-2 versus the same ester *sn*-1,3. This point was, therefore, clarified by using a selection of synthetic triglycerides.

### *4.2. Hydrolysis of synthetic lipids catalysed by CPGL*

The use of natural fats as substrates showed that CPGL seemingly exhibited no regio-selectivity for either the primary (*sn*-1 and *sn*-3) or secondary (*sn*-2) ester bonds of a triglyceride. However, the fact that the proportions of the fatty acids released from POP are almost identical to those released from PPO confirms the non-specific positional selectivity of CPGL. The proportion of the short-chain butyric acid released from the *sn*-2 position of PBP is much higher than that of the long-chain oleic acid released from the *sn*-2 position of POP, both after a 2 h period of catalysed hydrolysis, a result consistent with short-chain fatty acid ester preference of CPGL for catalysed hydrolysis.

Milk fat has almost 100% short-chain fatty acid (C4:0) occupying the *sn*-3 position, which is hydrolysed with a rate constant equal to $1.56\,h^{-1}$. With the PBP substrate, the short-chain ester (C4:0), positioned at *sn*-2, was hydrolysed with only a slightly lower rate constant equal to $1.14\,h^{-1}$, indicating that CPGL is relatively indifferent to the location of the short-chain ester on the glycerol backbone, and will attack a short-chain ester in preference to a long-chain one, no matter where it is located. This result is in complete contrast to that obtained for the lamb pregastric lipase catalysed hydrolysis of tributyrylglycerol [13] which showed that the ratio of the rate constants of hydrolysis of the butyrate ester from the *sn*-3 to *sn*-2 positions was $\sim$25:1, and the mechanism for hydrolysis of the C4:0 ester at *sn*-2 was not by direct hydrolysis, but by a prior acyl transfer of the ester to *sn*-3. Such positional non-selectivity, as identified in

this investigation, is extremely rare. Pancreatic lipase does catalyse the hydrolysis of esters on all three positions, but shows a strong preference for the primary over the secondary positions [14].

The value of the rate constant of catalysed hydrolysis for the palmitate esters located at *sn*-1,3 was too small (see Fig. 3 which yielded a rate constant equal to $0.0097\,h^{-1}$) to be identified within the time scale used for hydrolysis of PBP. However, the data shown in Fig. 5 confirm the relevant magnitudes of the rate constants for hydrolysis of C4:0 versus C16:0 lipid esters. The conclusion is, then, that CPGL exhibits, at best, only slight regio-selectivity, with perhaps a small preference for catalysing a lipid–ester located at *sn*-3 over the same ester located at *sn*-2.

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