Food Chemistry **22** (1986) 297–303

Large Agarose–Lipase Beads for the Hydrolysis of Triglycerides

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(Received 20 December 1985; accepted 3 April 1986)

ABSTRACT

Candida cylindracea *lipase could be immobilized by binding to large agarose beads or by entrapment within polyacrylamide polymer. Large agarose beads, prepared from native agarose, were preferred for their higher binding capacity and easy intact separation for reuse. The agarose–lipase complex is stable for 6 months at 4°C, maintaining 80% of its initial activity despite eight-fold re-use. Consequently, the lipase complex is recommended for semi-continuous hydrolysis of olive oil and milk fat triglycerides. The extent of total hydrolysis was 30·8 ± 1·25% for olive oil as against 23·1 ± 0·95% for milk fat.*

INTRODUCTION

The use of lipase-interesterification for the production of fats and oils with desirable characteristics was recently reported (Coleman & Macrae, 1980; Tanaka & Ono, 1981; Macrae, 1983). Moreover, the use of lipases as catalysts in monoglyceride production was reported by Nielsen (1985). In the processing of fats and oils for human consumption, the flavour can be changed or enhanced by partial hydrolysis of the lipid components. So far, pancreatin has generally been used as a source of lipase. However, the recent price increase of pancreatin has led to a renewed interest in immobilized lipases.

In the past few years there were far fewer detailed studies on the application of immobilized lipases to food industries (Suzuki & Karube,

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1977; Kobayashi *et al.*, 1980; Yamana *et al.*, 1982; Kimura *et al.*, 1983) than other enzymes. Thus, this study describes the immobilization of *Candida cylindracea* lipase and its possible reuse for natural triglyceride hydrolysis.

MATERIALS

Chemicals

Candida cylindracea lipase (16 U/mg protein), acrylamide, bisacrylamide and TEMED were supplied by the Sigma Chemical Co., St. Louis, Mo., USA. Sepharose 4B was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Milk fat and olive oil triglycerides were prepared from anhydrous milk fat and olive oil by preparative thin-layer chromatography. All other chemicals were reagent grade.

METHODS

Copolymerization of *Candida cylindracea* lipase

Preparation of lipase–polyacrylamide polymer was carried out according to Bernfeld & Wan (1963). The lipase was dissolved in 26·7 ml of 0·005M tris-HCl buffer (pH 7·5) to which 11·7 ml of 22% cyanogum and 0·3 ml TEMED were added. Then 14 mg ammonium persulphate were added and polymerization was performed under nitrogen at room temperature. The insoluble synthetic polymer formed was dispersed mechanically and washed several times with the above buffer and finally lyophilized.

Covalent binding of *Candida cylindracea* lipase

Large agarose beads (*ca.* 5 mm) were prepared from washed melted Sepharose 4B (water-bath at 90°C) by dropwise injection into an ice-cold mixture of toluene–chloroform–*n*-hexane (5:2:1, v/v/v). Excess solvent was removed by shaking twice in 1,4-dioxane, followed by successive washing in water. In principle, large agarose beads can be prepared by the method described above from any agarose gel. The agarose–water ratio producing viable beads was studied. Beads of $<3\%$ by weight of agarose are very easily broken up even when gentle stirring is employed. Very hard beads are obtained from 8% by weight of agarose. Concentrations higher than 8% produced a gel which does not melt into a homogeneous liquid as

required for the beading process. It would appear that a gel containing 4% to 6% agarose provides a suitable material for the production of large beads.

Agarose beads were activated with cyanogen bromide according to Axén *et al.* (1967). Immobilization of soluble lipase was performed at pH 8 for 4 h at 4°C. The beads were separated and washed with water and suspended in 100 mM ethanolamine adjusted to pH 8 with hydrochloric acid. Incubation was carried out for 2 h to ensure that any activated sites not available to enzyme protein were saturated. Finally, the beads were washed with a gradient of 0·1 to 1M phosphate buffer (pH 7·4) to remove unbound lipase and ethanolamine.

Assay of bound protein

Four grams of activated and neutralized beads were lyophilized. The dry immobilized lipase preparations were suspended with homogenization in 0·005M tris-HCl buffer (pH 7·5) and allowed to sediment. The absorbance was measured at 280 nm and protein concentration was calculated from the extinction coefficient of lipase protein at 280 nm.

Assay of lipase activity

Lipase activity was determined by titration of liberated fatty acids from olive oil emulsion according to Dole & Meinertz (1960). One lipase unit (U) was defined as the amount of enzyme which liberates 1 μM fatty acids per minute.

Hydrolysis of milk fat and olive oil triglycerides

The hydrolysis of milk fat and olive oil triglycerides was accomplished in a semi-continuous reactor with a recycling system at pH 7·5 and 35°C. The lipid emulsions were prepared by blending 4 g substrate, 4 g Triton X-100, 0·2 g $CaCl_2$, 0·234 g NaCl and the volume was made to 100 ml with 0·2M tris-HCl buffer (pH 7·5). The lipid emulsions were homogenized before use and the hydrolysis was performed for 30 min using 30U immobilized lipase per 2 ml substrate emulsion. The percentage hydrolysis was determined by thin-layer chromatography and scanning reflectance techniques. The reaction products were extracted with diethyl ether and fractionated on silica gel G type 60 plates (200 × 200 × 0·5 mm) in petroleum ether–diethyl ether–acetic acid (70:30:2, v/v/v). Spot visualization was achieved by spraying with H_2SO_4 (50%) and heating at 180°C for 10 min. The charred spots were measured with a densitometer (Shimadzu

CS-190) by reflectance scanning. This method revealed a positive relationship between the amount of spotted sample in the range 10–50 μg and light absorbance.

RESULTS AND DISCUSSION

The immobilization of *Candida cylindracea* lipase within polyacrylamide gel or on to agarose beads was carried out. The activities of lipase used in coupling, bound enzyme yield and actual enzyme activity were determined for both supports (Tables 1 and 2). For both carriers, bound

TABLE 1
Effect of Initial Lipase Activity on Bound and Actual Enzyme Activity of Agarose Lipase Beads

Experiment No.	*Added protein (mg)*	*Added activity (U)*	*Activity in washings (U)* $\bar{x} \pm SD$[a]	*Bound (A) lipase activity/4 g agarose beads* $\bar{x} \pm SD$	*Actual (B) lipase activity/4 g agarose beads* $\bar{x} \pm SD$	*(B)/(A) (%)* $\bar{x} \pm SD$
1	15·36	226	110 ± 5·77	116 ± 5·89	28·0 ± 1·41	24·1 ± 1·41
2	23·04	330	150 ± 9·13	180 ± 9·13	45·0 ± 2·16	25·0 ± 1·15
3	31·99	444	170 ± 9·63	274 ± 13·95	80·0 ± 2·94	29·2 ± 0·98
4	38·40	550	190 ± 10·80	360 ± 18·26	100·0 ± 4·4	27·8 ± 0·93

[a] $\bar{x} \pm$ SD, $n = 4$.
Bound and actual lipase activities are the activities/4 g agarose beads.

and actual lipase activities increased by increasing initial lipase activity used in coupling. However, similar lipase activity percentages for either supporting media were obtained at different enzyme concentrations. Whereas Lieberman and Ollis (1975), using polyacrylamide gel, obtained a maximum of 2·1 mg bound lipase protein per gram of polyacrylamide, Kilara *et al.* (1977) reported 17 mg bound lipase protein per gram of dry agarose. In contrast, in the present study, the amounts of 27 and 157 mg lipase protein per gram of dry support were obtained using polyacrylamide and agarose beads, respectively.

Similar pH and temperature optima values (Figs 1 and 2) were obtained for both soluble and immobilized lipase complexes (35°C and pH 7·5). Kobayashi *et al.* (1980) detected no change in either pH or temperature optima of *Rhizopus delemar* and *Pseudomonas fluorescens* lipases immobilized on Sepharose 4B. However, other reports have indicated shifts in pH and temperature optima of other lipases upon immobilization. Such effects

TABLE 2
Effect of Initial Lipase Activity on Bound and Actual Enzyme Activity of Polyacrylamide–Lipase Polymer

Experiment No.	*Added protein (mg)*	*Added activity (U)*	*Activity in washings (U)* $\bar{x} \pm SD$[a]	*Bound (A) lipase activity/g polymer* $\bar{x} \pm SD$	*Actual (B) lipase activity/g polymer* $\bar{x} \pm SD$	*(B)/(A) (%)* $\bar{x} \pm SD$
1	11·14	179	67·0 ± 3·92	107 ± 5·29	33·0 ± 1·41	30·8 ± 1·55
2	14·78	231	48·0 ± 2·94	183 ± 12·07	54·0 ± 2·58	29·0 ± 1·53
3	23·04	360	76·0 ± 3·65	284 ± 12·95	83·0 ± 4·24	29·2 ± 1·41
4	33·20	520	100 ± 5·35	420 ± 18·25	126 ± 5·48	30·0 ± 1·41

[a] $\bar{x} \pm SD$, $n = 4$.
Bound and actual lipase activities are the activities/g lyophilized polymer.

were observed with *Mucor* lipase (Ogiso *et al.*, 1972), pancreatic lipase (Melius *et al.*, 1976) and *Geotrichum candidum* lipase (Kroll *et al.*, 1980).

The apparent K_m values for soluble and immobilized lipase complexes derived from the Lineweaver–Burk plot (Fig. 3) indicated the values 5×10^{-2}, 2×10^{-2} and $1{\cdot}75 \times 10^{-2}$ M/litre^{-1} for polyacrylamide–, agarose–lipase complexes and soluble enzyme, respectively. The observed increased K_m value of *Candida cylindracea* lipase, as a result of rendering the enzyme insoluble, agrees with that for polyacrylamide–asparaginase (Mori *et al.*, 1973), which was attributed to the decrease of substrate concentration in the gel. In contrast, Melius *et al.* (1976) and Kilara *et al.* (1977) found that the apparent K_m values of the lipases decrease upon immobilization.

The simplicity of recovering lipase–agarose beads intact, and their higher binding capacity, indicated the superiority of this preparation over the

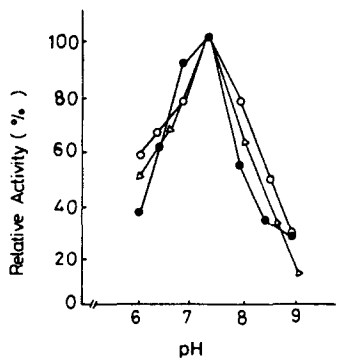

Fig. 1. pH profiles of free (●——●), agarose (△——△) and polyacrylamide–lipase (○——○).

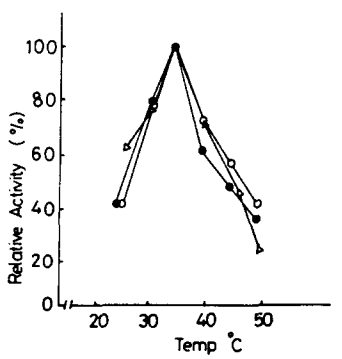

Fig. 2. Temperature activity profiles of free (●——●), agarose (△——△) and polyacrylamide–lipase (○——○).

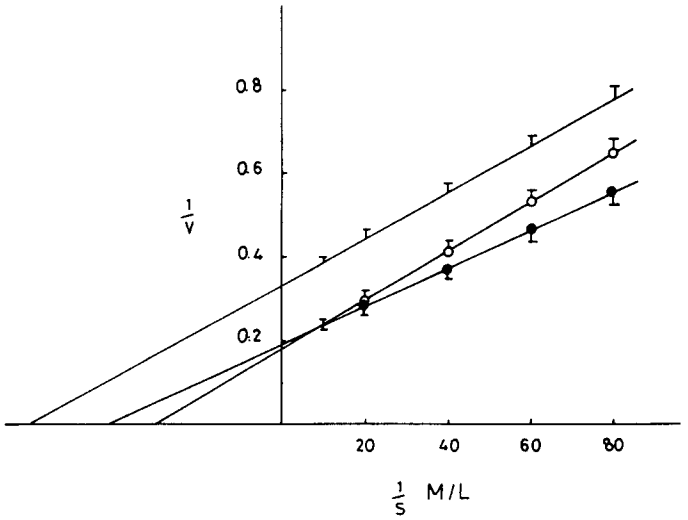

Fig. 3. Lineweaver–Burk plot for hydrolysis of olive oil triglycerides. Free lipase (———). Agarose–lipase (●——●). Polyacrylamide–lipase (○——○).

copolymerized lipase complex for the hydrolysis of natural triglycerides. This preparation was found to retain 80% of its initial activity after eight repeated uses and showed stability for 6 months at 4°C and pH 7·5. The amount of total hydrolysis of olive oil triglycerides was $30{\cdot}8 \pm 1{\cdot}25\%$ as against $23{\cdot}1 \pm 0{\cdot}95\%$ for milk fat (Table 3). The observed difference in total hydrolysis value for both substrates may be due to the higher specificity of *Candida cylindracea* lipase towards oleic acid glycerol ester (Benzonana & Esposito, 1971). The mono + diglyceride yields could be increased if acyl migration during hydrolysis is minimized.

TABLE 3
Hydrolysis (%) of Milk Fat and Olive Oil Triglycerides with Agarose–Lipase Complex

Fraction	*Hydrolysis (%)*	
	Milk fat $\bar{x} \pm SD^a$	*Olive oil* $\bar{x} \pm SD$
Monoglycerides	$3{\cdot}1 \pm 0{\cdot}81$	$4{\cdot}8 \pm 0{\cdot}51$
Diglycerides	$5{\cdot}5 \pm 0{\cdot}93$	$5{\cdot}2 \pm 0{\cdot}54$
Free Fatty Acids	$14{\cdot}5 \pm 0{\cdot}93$	$20{\cdot}8 \pm 1{\cdot}45$
Triglycerides (residual)	$76{\cdot}9 \pm 1{\cdot}87$	$69{\cdot}2 \pm 2{\cdot}55$
Total hydrolysis	$23{\cdot}1 \pm 0{\cdot}95$	$30{\cdot}8 \pm 1{\cdot}25$

[a] $\bar{x} \pm$ SD, $n = 6$.

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