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Low temperature organic phase biocatalysis using cold-adapted lipase from psychrotrophic *Pseudomonas* P38

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Lipase produced by a psychrotroph, *Pseudomonas fluorescens* P38, was found to catalyse the synthesis of butyl caprylate in n-heptane at low temperatures. The optimum yield of ester synthesis was 75% at 20°C with an organic phase water concentration of 0.25% (v/v). The results are discussed in terms of the structural flexibility of psychrotroph-derived lipase and the activity of this enzyme within a nearly anhydrous organic solvent phase. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Psychrotrophic microorganisms produce enzymes that are associated with spoilage and quality deterioration in refrigerated foods and dairy products (Dring & Fox, 1983; Stead, 1986; McKellar, 1989). Recently, it has become evident that enzymes from psychrotrophs and psychrophiles have some potential for low temperature catalysis at 0–20°C where the activity of mesophilic enzymes tends to be reduced (Gounot, 1991; Feller *et al.*, 1992).

Pseudomonas fluorescens P38 grows readily in milk in which medium lipase production is greater at 10°C compared to 25°C (Bucky *et al.*, 1987). Psychrotroph-derived lipases exhibit a high resistance to irreversible heat inactivation coupled with a low conformational stability. The origins of such unique heat inactivation properties are not fully understood but may be partly derived from the low enzyme conformational stability (Owusu *et al.*, 1991, 1992; Tan & Owusu Apenten, 1995, unpublished results).

The high specific activity of psychrotroph-derived enzymes, compared to the corresponding mesophile-derived enzymes, at low temperatures has been attributed to their relatively high conformational flexibility. At a temperature range of 5–25°C, amylase from the Antarctic psychrophile *Altermonas haloplanctis* A23 was found to have a catalytic efficiency that is 100-fold greater than that for an amylase from a mesophile (Feller *et al.*, 1994). Similar results have been widely reported for enzymes isolated from fish (Bjarnason & Asgeirsson, 1993). The flexibility of psychrotroph-derived, cold-adapted enzymes could make such enzymes particularly suited to low temperature biocatalysis.

In this paper the use of cold-adapted lipase from a psychrotrophic microorganism, *Psuedomonas flourescens* P38 (P38 lipase), for ester synthesis within an organic solvent phase is examined. Lipase-catalysed ester synthesis within organic solvent phases have been widely reported in recent years (Zaks & Klibanov, 1984; Klibanov, 1986; Dordick, 1989). However, there has been no previous systematic study of the use of psychrotroph-derived enzymes for low temperature organic phase biocatalysis. The objective of this study is to investigate the possibility of using a psychrotroph-derived lipase for the synthesis of a model ester (butyl caprylate). Caprylic acid esters have a fruity flavour and are present as flavour ester components in several fruits (Van Straden *et al.*, 1979). The principles illustrated may be useful in the enzymatic synthesis of heat-sensitive chemicals.

MATERIALS AND METHODS

Butanol, caprylic acid, n-heptane and all other chemicals were purchased from Sigma Chemical Co. (UK).

P38 lipase preparation

Lipase was produced from *Pseudomonas fluorescens* P38 culture grown in a minimal medium consisting of 2-[bis(2-hydroxyethyl)-amino]ethanesulfonic acid (25 mM), sodium pyruvate (50 mM), arginine (10 mM), potassium phosphate (5 mM), magnesium sulphate (1 mM),

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calcium chloride (1 mM) and iron chloride (20 mM); the medium was adjusted to pH 7.0 before use. The growth temperature and growth time were 20°C and 48 h, respectively. Cultures were centrifuged at 12000 rpm and the supernatants collected and dialysed against Tris–HCl (0.1 M, pH 8.0) at 5°C overnight followed by freeze-drying. The freeze-dried cell-free extract was used as the source of crude lipase without further treatment.

P38 lipase-catalysed synthesis of butyl caprylate in n-heptane

Ester synthesis was carried out in n-heptane (5 ml) containing butanol (0.25 M), caprylic acid (0.25 M) and crude lipase powder (0.1 g). All solvents and substrates were dried by storing over molecular sieve 3A overnight before use. Variable amounts of water (0–2.0%, v/v) were added to the n-heptane phase in order to examine the effect of water content on ester synthesis. The reaction mixture was incubated at pre-selected temperatures with constant shaking using a Stuart shaker (Stuart Ltd., UK) for at least 48 h.

The formation of butyl caprylate was monitored using a gas chromatograph (Model 92; Analytical Instruments Ltd., UK) fitted with a flame ionization detector and a DEGS (10%)/Chromosorb (80–100 mesh) column (Supelco Ltd., UK).

P38 lipase heat stability in n-heptane

Equal amounts of dry lipase were suspended in (1) n-heptane or (2) n-heptane with 0.25% (v/v) added water; the stability of lipase (3) dissolved in water was also examined. The enzyme samples were incubated at 10–60°C for 48 h and cooled using an ice bath. In cases (1) and (2) the organic solvent was subsequently removed by gassing with nitrogen at room temperature and the dry lipase redissolved in an equal volume of buffer (0.05 M Tris–HCl buffer, pH 7). In all cases the remaining lipase activity was assayed spectrophotometrically using *p*-nitrophenyl caprylate as substrate (Winkler & Stuckman, 1979).

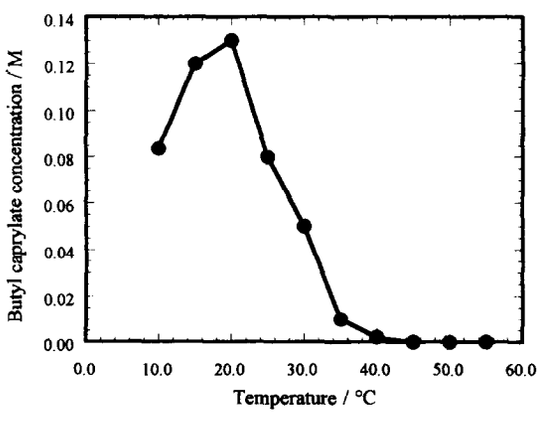

Fig. 1. Effect of temperature on butyl caprylate synthesis over a period of 48 h.

RESULTS AND DISCUSSION

Effect of temperature on ester synthesis and P38 lipase stability in n-heptane

The use of lipase from *Pseudomonas* spp. for ester synthesis has been reported at temperatures of 30–50°C (Langrand *et al.*, 1988; Welsh *et al.*, 1990; Shaw & Lo, 1994). Porcine pancreatic lipase catalysed ester synthesis at 100°C (Zaks & Klibanov, 1984). However, the temperature-dependence of enzymatic organic phase ester synthesis has not been discussed at any length.

Figure 1 shows the temperature optimum for butyl caprylate synthesis, using P38 lipase, to be 20°C. The decrease in ester synthesis above 20°C is most likely due to lipase inactivation. To examine this issue the heat stability of P38 lipase suspended in n-heptane was examined at 10–60°C.

Figure 2 shows the residual activity of P38 lipase samples after heating at 10–60°C for 48 h. Lipase dissolved in water was found to be least stable (Fig. 2, line 3). In comparison, the dry P38 lipase was stable until 40°C but became increasingly inactivated at higher temperatures (Fig. 2, line 1). Enzyme inactivation within anhydrous organic solvents proceeds via the same mechanisms as inactivation processes in aqueous solvents. There is enzyme unfolding followed by one or more irreversible chemical changes. The irreversible inactivation step involves protein aggregation within dry organic solvents. The rate of inactivation is usually greatly reduced for dry enzymes due to a decrease in water activity within the dry organic phase (Volkin *et al.*, 1991).

With the addition of 0.25% (v/v) water to the n-heptane phase (the system used for synthesis), lipase inactivation occurred at temperatures above 20°C (Fig. 2, line 2). Thus it may be inferred that P38 lipase inactivation led to the decrease in ester synthesis above 20°C (Fig. 1). A low thermal stability is often regarded as the consequence of the flexible conformational structure of cold-adapted enzymes (Feller *et al.*, 1990; Jaenicke,

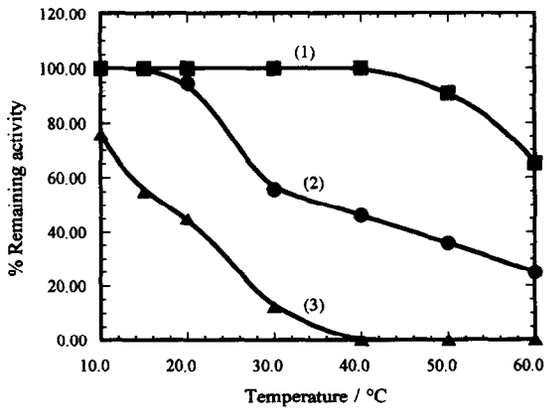

Fig. 2. Comparison of lipase stability in (1) n-heptane, (2) n-heptane with 0.25% (v/v) water, and (3) water. Lipase was incubated in these systems at the respective temperatures for 48 h before activity was assayed and expressed as a percentage of the original activity.

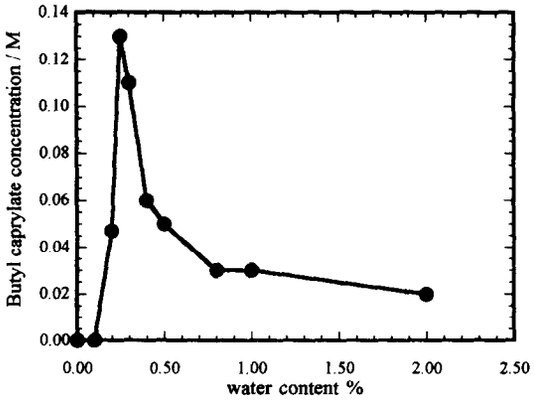

Fig. 3. Effect of added water on butyl caprylate synthesis. Reaction medium was incubated at 20°C for 48 h.

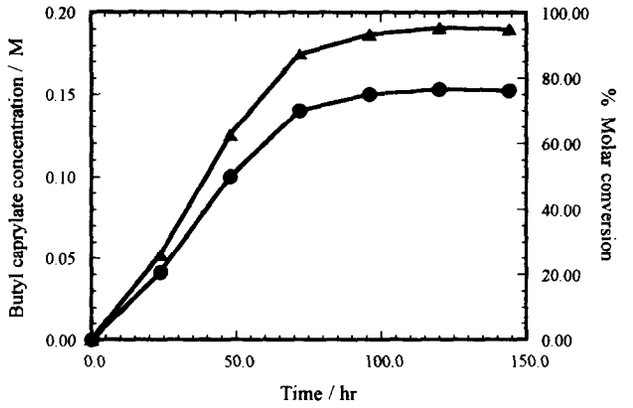

Fig. 4. Time course of the synthesis of butyl caprylate catalysed by lipase P38 at 20°C. Both ester concentration (▲) and percentage yield (●) are shown.

1990; Feller *et al.*, 1994). In general, thermostability within an organic solvent will be achieved if the enzyme is intrinsically rigid, or if the environment (e.g. low water activity) prohibits enzyme flexibility. Enzymes that are stable/rigid within organic solvent phases are also likely to exhibit low specific activity at low temperatures. On the other hand, high specific activity within solvents may be associated with a highly flexible and relatively heat-labile enzymes (Owusu & Cowan, 1989, 1990).

Effect of water content on P38 lipase catalysed ester synthesis in n-heptane

The possible inverse relationship between enzyme stability and activity within organic solvents was examined indirectly, by assessing the effect of organic phase water content on ester synthesis. A minimum amount of water may be essential to produce a degree of enzyme flexibility necessary for catalysis (Klibanov, 1986; Zaks & Russell, 1988; Dordick, 1989). Figure 3 shows that the yield of butyl caprylate was optimum at an organic phase water concentration of 0.25% (v/v). At a higher or lower water concentration the yield of ester decreased.

The decrease in butyl caprylate yield at a high water concentration might be due to the increased tendency for lipase to undergo heat inactivation as the amount of water in n-heptane is increased (Fig. 2). A high concentration of water would also shift the product–reactant equilibrium towards ester hydrolysis, leading to a decrease in butyl caprylate yield. However, a decrease in butyl caprylate yield at a low organic phase water concentration ($< 0.25\%$, v/v) cannot be explained in these terms. P38 lipase was relatively stable in dry n-heptane (Fig. 2). Also a low water concentration can be expected to shift the reactant–product equilibrium towards ester synthesis. It may be concluded that, in a completely dry organic solvent phase, P38 lipase (although stable) does not possess sufficient flexibility to enable the catalysis of butyl caprylate synthesis.

The optimum amount of water required for organic phase biocatalysis may depend on factors such as the type of organic phase and choice of enzyme (Laane *et al.*, 1986; Zaks & Russell, 1988). It is not yet possible to generalize as to whether psychrophile-derived enzymes, in view of their proposed flexibility, require a greater concentration of organic phase water for catalysis. However, an organic phase water content of 0.1–0.6% (v/v) has commonly been adopted by other workers (Zaks & Klibanov, 1984; Klibanov, 1986; Manjon *et al.*, 1991; Shaw & Lo, 1994; Langrand *et al.*, 1988).

Time course of ester synthesis using P38 lipase in n-heptane

The reaction catalysed by the P38 lipase reached an equilibrium after 96 h at 20°C with a final molar conversion of 75% (Fig. 4). Compared to reactions in most biphasic systems (Dias *et al.*, 1991; Boreix *et al.*, 1992) which reached equilibrium before 24 h, this is a considerably slower reaction. Low temperature organic phase biocatalysis is expected to be associated with a slower rate of reaction and higher organic solvent phase viscosity. Further studies are necessary to establish the degree to which the above factors can be compensated for by the expected higher intrinsic activity of psychrophile-derived enzymes.

CONCLUSIONS

Cold-adapted lipase extracted from psychrotrophic *Pseudomonas fluorescens* P38 catalysed ester synthesis in n-heptane at temperatures of 10–35°C. Optimum synthesis occurred at 20°C in the presence of a 0.25% (v/v) water in the organic phase. By considering the stability of cold-adapted lipase in organic solvents containing varying amounts of water, it is possible to relate the structural flexibility and activity of P38 lipase indirectly to the yield of butyl caprylate synthesis in n-heptane. Reaction conditions have to be such that a required degree of enzyme flexibility as well as stability is obtained. This work illustrates the possibility of using cold-adapted enzymes from psychrotrophs for low

temperature biocatalysis. The higher conformational flexibility and high specific activity of psychrophile-derived enzymes may be advantageous in organic phase biocatalysis. Low temperature organic phase biocatalysis might in future have applications in the preparation of heat-sensitive, high value, products.

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