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Effect of nonionic detergents on the activity of a thermostable lipase from *Bacillus stearothermophilus* MC7

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Abstract

The influence of nonionic surfactants on the activity of a novel thermostable lipase from *Bacillus stearothermophilus* MC7 was investigated with a view to its potential for synthesis of structured lipids. A large number of modifiers within a broad concentration range were applied. The activity of the enzyme was measured at a relatively high reaction temperature. Highest degree of activation was observed when PEG_{6000} was applied (up to 2.3-fold increase). Modification essentially changed the performance of the lyophilised preparations—they keep up to 80% of the activity of the native enzyme in the presence of a detergent against 30% in its absence. The effect of sorbitan esters (spans) and polyoxyethylene derivatives of sorbitan esters (tweens) on lipase MC7 was estimated, their HLB value varying within the interval 2.1–16.7. Tweens were strong inhibitors at higher concentrations. For all spans, excepting span 60, an increase of enzyme activity with concentration was observed. All studied additives slow down the process of thermal denaturation. Lipase preparations preserve more than 60% of their activity after 30-min incubation at 75 °C in the presence of tween 60 or PEG_{4000}.

Keywords: *Bacillus stearothermophilus*; Thermophilic lipase; Activation; Freeze-drying; Nonionic detergents

1. Introduction

The biological function of lipases (EC 3.1.1.3) is to catalyse the hydrolysis of acylglycerols to free fatty acids and glycerol. In industry they are applied for synthesis of structural triglycerides, which are used as detergents and emulsifiers, in clinical nutrition and cosmetics [1–3]. They also find application in kinetic resolution of racemic mixtures [4,5], and for modification of the physical and functional properties of oils and fats [6]. The drawbacks of the industrial application of lipases are their expensive production and low stability, which can be overcome by exploring new sources, by immobilisation and activation of the biocatalyst. Different bio (polypeptides and polysaccharides) and synthetic (polyethylene glycols, polypropylene glycols, crown ethers and their derivatives) polymers act as modifiers and influence the efficiency of catalysis by different mechanisms. They can either stabilise the globular proteins and prevent them from forming aggregates or increase substrate solubility. They can solve the problem with solvent inhibition and preserve enzyme activity in organic media [7–11]. Some modifiers alter enzyme specificity [12].

In the present article, we report the influence of three series of nonionic detergents on the stability and catalytic activity of a newly isolated thermostable lipase from *Bacillus stearothermophilus* MC7 [13,14]. We estimated their effect at relatively high reaction temperature in a broad concentration range. All studied additives contribute for the thermal stability of the enzyme. The surfactants also proved to be indispensable in freeze-drying of lipase MC7, which is the basis for further optimisation of the biocatalyst for synthetic reactions.

2. Materials and methods

2.1. Materials

Lipase from *B. stearothermophilus* MC7 (lipase MC7) was isolated and purified according to the method described by Kambourova et al. [14]. Poly(ethylene glycol) (PEG) with differ-

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ent molecular weights, polyoxyethylene sorbitan monolaurate (tween 20), polyoxyethylene sorbitan monopalmitate (tween 40), polyoxyethylene sorbitan monostearate (tween 60), polyoxyethylene sorbitan monooleate (tween 80), polyoxyethylene sorbitan trioleate (tween 85), sorbitan monolaurate (span 20), sorbitan monopalmitate (span 40), sorbitan monostearate (span 60), sorbitan tristearate (span 65), sorbitan monooleate (span 80) and 4-nitrophenyl palmitate (98% purity) were purchased from Fluka (Germany).

2.2. Lipase activity assay

The lipase activity was estimated to be 1400 lipase units per millilitre (specific activity 350,000 Ug^{-1}), using a spectrophotometric assay with 4-nitrophenyl palmitate as a substrate [15]. One enzymatic unit is equal to the quantity of enzyme liberating 1 μmol 4-nitrophenol per minute. Its molar absorption coefficient was determined to be 1.46×10^5 cm^{-1} M^{-1} at 410 nm. The reaction temperature was 60 °C. The reactions were followed on spectrophotometer Shimadzu UV/vis 3000 in triplicate with standard deviation ± 5%. Relative activity is calculated with respect to the activity of the native enzyme.

2.3. Lipase pretreatment

One millilitre of the diluted lipase solution (14 U/mL; specific activity 3500 Ug^{-1}) was mixed with a detergent solution. The concentrations of poly(ethylene)glycols varied in the interval from 10^{-7} to 10^{-3} M. The concentrations of the sorbitan derivatives were in the interval 2.7×10^{-5} to 2.7×10^{-3} M. The preparations were gently agitated for 2 h and kept for 12 h at 4 °C. Freeze-dried preparations were obtained after 2-h incubation of 1 mL of the lipase solution with 0.5 mg PEG at room temperature.

2.4. Thermostability

One millilitre of the diluted lipase solution (14 U/mL; specific activity 3500 Ug^{-1}) was preincubated with the detergent at its optimal concentration at different temperatures within the range of 50–100 °C for 30 min followed by activity assay at 60 °C as described in Section 2.1.

3. Results and discussion

PEGs with molecular weight varying from 400 to 70,000 Da are the first series of surfactants we applied to lipase MC7 (Fig. 1). The concentration was varied within five orders of magnitude, since lipase activity is scarcely affected by the degree of modification.

Activation is observed for all PEGs used. PEGs with high degree of polymerisation have stronger effect at lower concentrations, and those with low degree of polymerisation are better activators at higher concentrations. We achieved maximal 2.3-fold activation with PEG_{6000} at concentration of the same order of magnitude as the concentration of the enzyme.

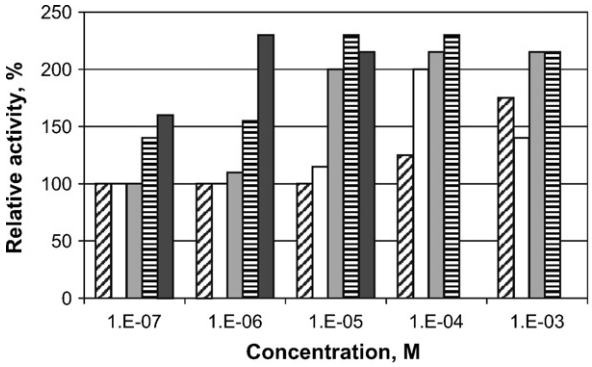

Fig. 1. Effect of PEGs on lipase MC7 activity. Additives: (▨) PEG_{400} (□) PEG_{2000} (□) PEG_{4000} (▩) PEG_{6000} (■) $PEG_{70,000}$.

Due to lack of data on the properties of thermostable lipases we analysed our results comparing them with the available crystallographic and kinetic data about some mesophilic lipases. Crystallographic studies have proven that lipases undergo conformational rearrangements at the water/oil interface making the active site accessible for the substrate molecule. Some authors compare the role of PEG to the one of the organic phase in the biphasic system. The surfactant and the enzyme form a complex with the aliphatic chain of the amphiphile exposed to the solvent and the hydrophilic moiety is completely buried in the lipase active site pocket. In this complex the enzyme is stabilised in its active "open" conformation [16–21]. These modifiers ensure optimal orientation and/or hydration state of the lipid [17,22].

This 2.3-fold increase of activity is comparable with the results for another bacterial lipase. *Candida rugosa* lipase is a mesophilic enzyme and like lipase MC7, has a broad substrate specificity. Their molecular weights are around 60 kDa. Soares et al. [23] observed five-fold increase in activity of the lyophilised preparation after modification of *C. rugosa* lipase with PEG_{1500}. Mine et al. [20] reported no activation with PEGs with low molecular weight, for PEG_{4000}, 1.2-fold, and for PEG_{2000}, 2.5-fold increase of activity of *C. rugosa* lipase. Here, we observe a different dependence of the degree of activation on the degree of polymerisation of PEG.

Freeze-drying of lipase MC7 leads to significant loss of activity. Irreversible structural changes occur due to the removal of the water molecules maintaining the active form. Our activation pro-

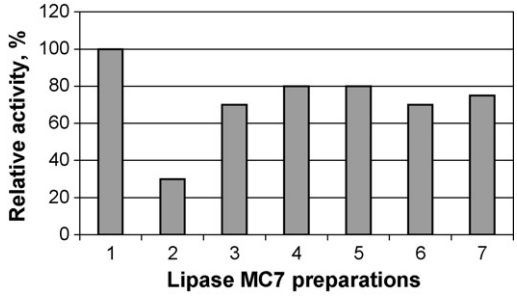

Fig. 2. Effect of PEGs on lipase MC7 activity after freeze-drying. Lipase preparations: (1) native enzyme (not lyophilised, no additive). Lyophilised: (2) no activator; (3) PEG_{400}; (4) PEG_{2000}; (5) PEG_{4000}; (6) PEG_{6000}; (7) $PEG_{70,000}$.

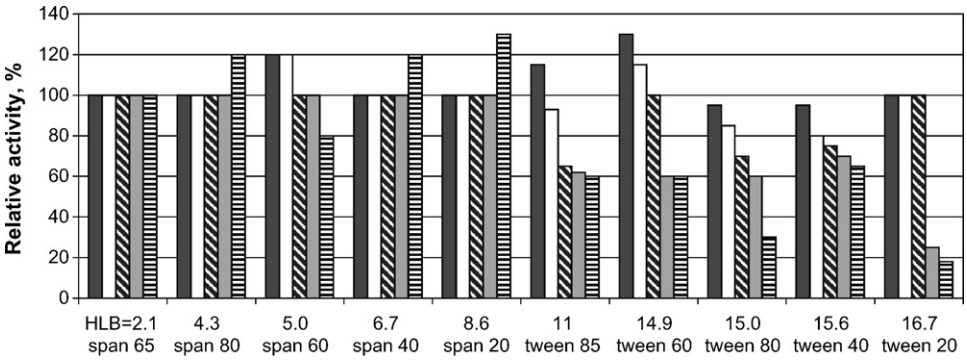

Fig. 3. Effect of sorbitan derivatives on the stability and activity of lipase MC7. Additive concentrations: (■) 2.7×10^{-5} M (□) 2.7×10^{-4} M (▤) 7.0×10^{-4} M (□) 1.4×10^{-3} M (■) 2.7×10^{-3} M.

cedure significantly improved the resulting preparations. After co-lyophilisation with PEG_{2000} or PEG_{4000} their activity was up to 80% of the activity of the native enzyme (Fig. 2). The surfactants stabilise the protein molecule as well as prevent aggregate formation in solution. Mingarro et al. [19] suggest that ligand binding induces in the enzyme a conformational change to active form which is preserved in the lyophilised sample when exposed to anhydrous solvent (ligand-induced enzyme "memory").

The effect of sorbitan esters (spans) and polyoxyethylene derivatives of sorbitan esters (tweens) on lipase MC7 was estimated (Fig. 3). We chose surfactants with hydrophilic–lipophilic balance (HLB) value varying within the interval 2.1–16.7. The HLB value indicates the polarity of the molecule in arbitrary units and its value increases with increasing hydrophilicity. Tweens (HLB from 11.0 to 16.7) were activators at low concentrations, which can be explained with improved substrate solubility. Higher surfactant concentrations lead to significant inhibition. This is attributed to excessive adsorption of the modifier on the enzyme surface resulting in diffusional limitation on the reaction.

For all spans, excepting span 60, an increase of enzyme activity with concentration was observed. In the biphasic reaction system they facilitate substrate solution reducing the water shell around the molecule, which leads to concentration gradient in the beginning of the reaction. Span 65 does not affect the activity of lipase MC7, but it still can contribute for the efficiency of the synthetic reaction by influencing enzyme stability and selectivity, and substrate solubility.

Having in mind the positive effect of the modifiers on enzyme activity, we examined their protectory properties at higher temperatures, water as media and the absence of a substrate. We preincubated the enzyme with the corresponding activator for 30 min at different temperatures (50–100 °C) and estimated the residual catalytic activity with *p*-nitrophenylpalmitate at 60 °C. The activators were added in their optimal concentration as found in the experiments so far: PEGs, 1×10^{-5} M and spans and tweens, 2.7×10^{-5} M. The activity of the native enzyme (no activator, no thermal pre-treatment) in the *p*-nitrophenylpalmitate test is taken for 100%. The results are presented in Table 1. As can be seen, thermal pre-treatment with-

Table 1
Effect of additives on lipase MC7 thermostability

Relative residual activity[a] (%)											
Additive	Temperature (°C)										
	50	55	60	65	70	75	80	85	90	95	100
Without activator	63.3	50.0	50.0	38.9	40.8	30.1	0.0	0.0	0.0	0.0	0.0
Span 20	68.2	51.0	60.0	46.9	60.0	40.0	16.7	10.0	5.5	4.4	4.7
Span 40	63.6	53.2	59.1	63.8	45.8	44.3	14.0	13.0	4.1	2.3	5.3
Span 65	61.0	51.8	64.4	52.7	39.7	31.5	14.5	12.5	6.5	7.6	5.0
Span 60	72.3	60.0	75.7	90.7	66.4	37.5	25.5	15.0	7.3	6.5	7.2
Span 80	58.0	55.0	60.0	52.0	45.0	36.6	15.0	14.5	3.7	5.2	4.9
Tween 85	73.4	76.7	54.8	43.1	50.0	30.0	13.8	13.1	3.4	18.0	18.0
Tween 20	57.5	51.7	50.0	46.8	55.7	29.3	12.7	10.0	7.8	18.7	21.4
Tween 40	55.6	58.1	51.1	44.4	61.7	42.2	15.0	10.5	7.4	24.0	17.0
Tween 60	85.0	92.4	81.9	82.7	58.7	63.9	17.7	18.0	16.3	22.6	21.6
Tween 80	67.8	64.3	55.0	48.0	56.0	40.0	14.5	13.5	6.8	21.5	19.8
PEG_{400}	95.0	125.0	95.0	82.0	65.0	40.0	25.0	15.7	11.5	7.5	4.9
PEG_{2000}	97.0	120.0	82.0	100.0	65.0	54.0	24.0	15.0	15.0	7.1	6.0
PEG_{4000}	86.0	125.0	95.0	90.0	66.0	68.0	25.0	28.0	16.8	13.6	4.7
PEG_{6000}	135.0	135.0	97.0	115.0	82.0	54.0	27.5	30.2	19.4	17.5	10.0
$PEG_{70,000}$	125.0	110.0	72.0	72.0	58.0	45.0	23.8	29.0	14.7	9.5	5.7

[a] The activity of the native enzyme (no additives, no thermal pre-treatment) is taken for 100%.

out additives leads to significant reduction in catalytic activity. The enzyme was completely denaturated at temperatures above 80 °C. However, the presence of all studied additives has a positive impact on enzyme stability. While with spans and tweens the residual activity is up to 50% compared to the native enzyme, using PEGs as modifiers we can even observe significant activation effect. It impressing that in the presence of additives the enzyme conserves about 20% of its activity at temperatures as high as 100 °C. Span 60, tween 60 and PEG_{6000} have the strongest protective effect of all studied additives.

Only few thermostable lipases from thermophilic microorganisms have been isolated recently. Most of the literature data concern enzyme expression and purification [24–27]. Only one crystal structure of a thermophilic lipase has been resolved [28]. There is scarcely any data about their application and stability in the presence of additives [29,30]. We present a broad analysis on the effects of three series of surfactants as a function of concentration, temperature, HLB value, molecular weight. The data from this first stage of our research on lipase MC7 combined with successful immobilisation techniques and knowledge of its specificity are indispensable for developing an efficient biocatalyst optimised for synthesis of structured lipids in non-conventional media.

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