Properties and Immobilization of *Enterobacter aerogenes* 13 Lipase

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Abstract

Lipases are biocatalysts of great importance due to their ability to catalyze diverse reactions. They are extensively used for the catalysis in organic solvents, which leads to multiple industrial applications.

The purpose of the present study was to estimate the main properties of soluble and immobilized Enterobacter aerogenes 13 lipase to establish a knowledge basis for implementing further biotechnological applications. Enterobacter aerogenes 13 strain was chosen for the research as a bacterial strain capable to hydrolyze lipids with a purpose to create the biochemical knowledge basis for further cheap and effective industrial lipase applications.

The free and immobilized ones on lignin and polyurethane lipase was determined to be the most active in mildly alkaline conditions (pH 8,0 – 9,0) and in the temperature range of 30 – 40 ºC. The enzyme was found to be stable in a wide pH range for long periods of time even in room temperature. No considerable improvement in thermostability has been achieved after immobilization, though lipase binding on solid support resulted in a slightly higher optimum temperatures and lower pH. Both, the free and the immobilized lipase preferred to hydrolyze medium chain-length fatty acid esters, also transesterification reactions were effective. The highest activity and protein yields were regained after covalent immobilization on Granocell® and polyurethane supports.

The results suggest that the first-time studied Enterobacter aerogenes 13 lipase is a perspective catalyst for hydrolysis, esterification and transesterification of various fatty acid esters.

Keywords: lipases, properties, solid supports, immobilized enzymes

Introduction

Lipases are amongst the most widely used biocatalysts due to their ability to catalyze diverse reactions [1, 2, 3,4]. Methyl esters of fatty acids, which are known as biodiesel can be produced by methanolysis of various triacylglycerols (TAG), such as plant oils (edible oils) and animal fats. The industrial-scale production of biodiesel fuel is performed by a chemical process using alkaline catalysts, fatty acid alkaline salts (soaps) are generated as by-products. They are removed by water washing. Hence, disposal of the resulting alkaline wash water creates other environmental concerns. Enzymatic production of biodiesel fuel from various fats is therefore strongly desirable and does not generate any waste materials. Biodiesel is pursued not only for the consideration of the future shortage of mineral supplies but also for the well-being of the environment as it is carbon-neutral. By utilizing the specific properties of lipolytic enzymes one can affect a selective transesterification reaction what is not possible in traditional chemically-catalysed transesterification. It was expected that the application of lipases could enable high conversion yields of oils and fats and simplify product purification at the same time [5, 6].

Materials and methods

Materials. Lipase from *Enterobacter aerogenes* 13 was kindly provided by JSC „BIOCENTRAS“, Vilnius, Lithuania. All chemicals used in the study were products of guaranteed grade. Oleic acid, triolein (TO), diolein (DO, mixture of 1,3-DO-85% and 1,2-DO-15%), monoolein (MO); fatty acids esters: p-nitrophenyl butyrate (p-NPB), p-nitrophenyl caprilate (p-NPC), p-nitrophenyl caprate (p-NPCa), p-nitrophenyl laurate (p-NPL), p-nitrophenyl mirystate (p-NPM) and p-nitrophenyl palmitate (p-NPP), were purchased from Fluka and Sigma; methyl, ethyl and 2-propyl alcohols, petrol- and diethyl ethers, n-hexane, acetic, boric, phosphoric and hydrochloric acids were purchased from Lachema and Roth, Tris was purchased from Serva; silica gel G-25 plates for thin layer chromatography (TLC) were purchased from Merck.

Methods

Partial purification of enzyme. Ammonium sulphate precipitation. Ammonium sulphate was added by ratios of 25, 40, 60, 80% saturation to *Enterobacter aerogenes 13* culture supernatant at 4ºC. The precipitate of every fraction was collected by centrifugation at 12000 x g at 4ºC for 20 Min and dissolved in 0.05 M phosphate buffer (pH 8.0). Lipolytic activity and protein concentration were determined by Bradford [7] and standard spectrophotometric assays. The purest fraction was defined by SDS-PAGE electrophoresis with 10% separating and 4% concentrating gels [8], and was further used for the experiments.

The standard spectrophotometric assay [9,10]. Hydrolytic activity of lipase upon p-NPB solution in 2-propanol was investigated measuring the change of optical density at 400-410 nm during 3-6 min at 30^0 C and pH 7.0-10.0, 100 mM universal buffer (ub) (Britton – Robinson buffer; composed of acetic, orto-boric and orto-phosphoric acids at a ratio of 1:1:1 providing buffering capacity over a wide range of pH [11,12]. One unit of lipase hydrolytic activity (HA) corresponds to the amount of the enzyme releasing 1 μmol of p-nitrophenol per minute under standard conditions.

Hydrolysis of *p*-NP fatty acid esters of various chain lengths by lipase. The rates of other *p*-NP fatty acid esters hydrolysis reactions were measured under the same conditions as of p-NPB. Solutions of p-NPC, p-NPCa, *p*-NPL and p-NPM in propan-2-ol were used as substrates.

Titrimetric assay [13] for the determination of lipolytic activity (LA) on emulsified substrates: the final concentration of oil was 10 % (v/v), gum arabic or polyvinyl alcohol (PVA) – 8.0% (v/v), triacylglycerols (TAG) - 10-50 mM. One unit of lipolytic activity corresponds to the amount of the enzyme releasing 1 μmol of fatty acid per minute under standard conditions.

Chromatographic analysis was carried out by thin layer chromatography method on silica gel G-25 plates. System of solvents for the elution: light fraction of petrol ether/diethyl ether/acetic acid (80:30:1 (v/v) was chosen according to [14] and our modification [15]. Chromatograms were developed by iodine vapor. Solutions of pure fatty acids and alcohols in diethyl ether were used as standards. Quantitative analysis (%) of reaction products separated by TLC (average of 3 assays) were performed using the micro image 4.0 program considering the spot area and colour intensity.

Transesterification reactions were conducted in thermostated 20 ml glass scintillation vials under shaking (180 rpm). Rapeseed oil and methanol were mixed in a molar ratio of 1:3 unless specified otherwise. Both soluble and immobilized lipases were used with an amount equivalent to 1 mg· g^{-1}. The reaction progress was followed by extracting 200–500 μl

aliquots of reaction mixture at definite time intervals and analyzing by TLC. For the analysis by TLC method samples were diluted with diethyl ether (v/v ratio 1:1) and kept at - 20ºC until the chromatographic analysis was carried out [15].

Lipase immobilization. A number of lignin, polyurethane (PU) and chitosan (CHS) derivatives were prepared and analyzed as supports for lipase immobilization in the Department of Polymer Chemistry, Vilnius University [16,17]. Polyurethane microparticles were synthesized from poly(vinyl alcohol) and hexamethylene diisocyanate according to. Carriers from chitosan and poly(ethylene glycol) methyl ether acrylate graft copolymers (CHS-GC) were obtained in homogeneous conditions using $(NH_4)_2Ce(NO_3)_6$ as an initiator and activated by using glutaraldehyde (GA) for covalent immobilization.

Magnetic derivatives of lignin and chitin (CHT) were prepared and analyzed as supports in the Department of Biochemistry and Biphysics, both of Vilnius University [18,19]. Granocell 2000 (Gr-2000) and Granocell 4000 (Gr-4000) were provided by Kaunas Technological University, Lithuania. Granocell, a macroporous cellulose (exclusion limit $2 \cdot 10^6$, wet bead size 160-315 mm) was synthesized according to [20]. Amino groups were introduced onto carrier by the action of epichlorhydrin and pentaethylenehexamine. The modified carrier was activated with glutaraldehyde for covalent immobilization.

Immobilization by adsorption. The suspension of supports and water solution of lipase was incubated for 3-24 h on a stirring plate. After immobilization, the preparation was vacuum filtered through a Buchner funnel and rinsed with appropriate buffer. The immobilizates were separated by vacuum filtration.

Soluble and immobilized protein yield and lipolytic activities were estimated by standard procedures as described above.

Equipment. Spectrophotometer 6405 UV/Vis with a thermostatically controlled cuvette (Jenway, UK); titration system Titronic 96 (Schott Glass Ltd. UK); pH-meter pH-720 (InoLab); thermostatically controlled magnetic stirrer (Ikamag); thermal stirrer TS-100 (Biosan); a vortex mixer (Fisher Vortex Genie 2TM USA); a system for electrophoresis (Merck); automatic micropipettes (Labsystems) and microsyringes (Hamilton).

Statistics. Four measurements were provided in each experiment and results are presented as means ± standard deviation.

Results and discussion

Effect of pH and temperature on Enterobacter aerogenes 13 lipase activity and stability

A comparative study between soluble and immobilized *E. aerogenes* lipase is provided in terms of pH and temperature. The enzyme was immobilized onto polymeric carriers by covalent binding. In the case of immobilization of lipase onto PU microparticles mostly covalent bonds between amino and hydroxyl groups of the enzyme and izocyanate groups of PU may cause immobilization. It is usually accepted that reactions between the carbonyl group of bifunctional agent glutaraldehyde and the amino groups of enzymes take place yielding Schiff bases. Due the porous structure of PU as well as graft CHS, adsorption of lipase or entrapment of enzyme into pores of microparticles could take place, too.

The pH and temperature profiles of lipolytic activities are shown in Figures 1 and 2, respectively. The partially purified soluble enzyme was the most active toward p-NPB in mildly alkaline pH range and showed optimum activity around pH 9.0. At pH 7.0 it's activity was only about 10% of the maximal activity, at pH 10.0 about 60% of maximal activity was preserved. A slightly lower optimum pH values were found for covalently immobilized on PU (pH 8.5) and magnetic lignin (pH 8.0) *E. aerogenes* lipase preparations, which also

exhibited a relatively higher activity at pH 7.0, showing 70% (lignin-lipase) and 57% (PU-lipase) of the maximal activity (Figure 1).

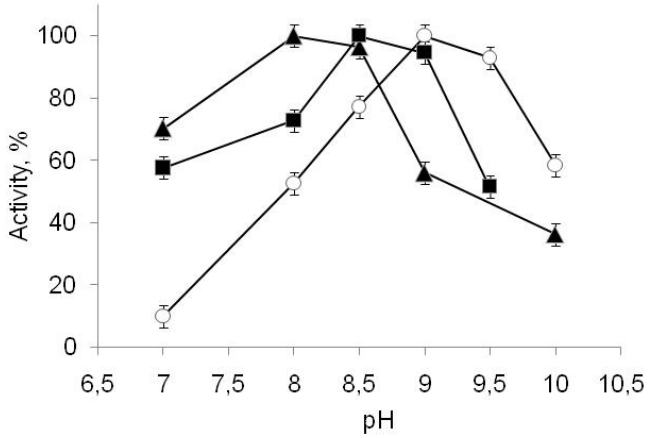

Figure 1. Effect of reaction pH on catalytic activities of soluble (○) and covalently immobilized on magnetic lignin (▲) and PU (■) *E. aerogenes 13* lipase preparations.

The optimal reaction temperature shifted from 35°C for the soluble lipase to 40°C for the lignin- and PU- immobilized lipase (Figure 2), implying that the immobilized lipase is more stable in higher temperatures. However thermal stability was not greatly increased after immobilisation, having 50% of residual activity after 8 min incubation at 60°C (similar for soluble lipase).

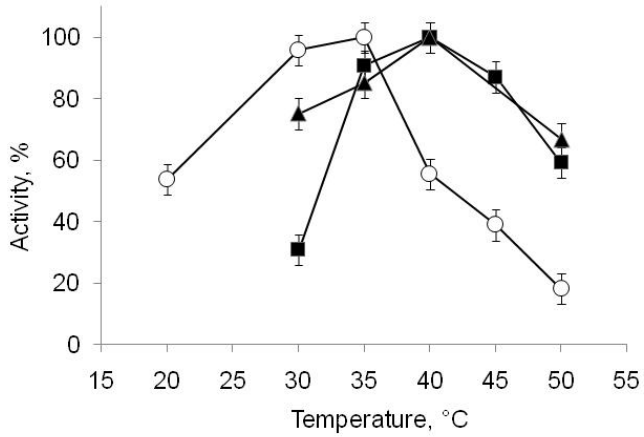

Figure 2. Effect of reaction temperature on catalytic activities of soluble (○) and covalently immobilized on magnetic lignin (▲) and PU (■) *E. aerogenes 13* lipase preparations.
Lipase was found to be relatively stable both at 20° and at 4°C and after 4 month preservation showed 44-47% residual activities in neutral pH ranges.

Substrate specificity of Enterobacter aerogenes 13 lipase

The enzyme specificity was studied with p-nitrophenyl alkanoate esters of varying alkyl chain lengths (C3-C14). The highest hydrolysis rates in case of soluble and immobilized lipase were obtained with p-NP-caprylate (C8), indicating enzymes preference for medium-size acyl chain lengths (Figure 3). The relative catalytic activities of immobilized enzymes were significantly higher than that of soluble in case of all other esters. Therefore immobilization broadens the enzyme specificity range.

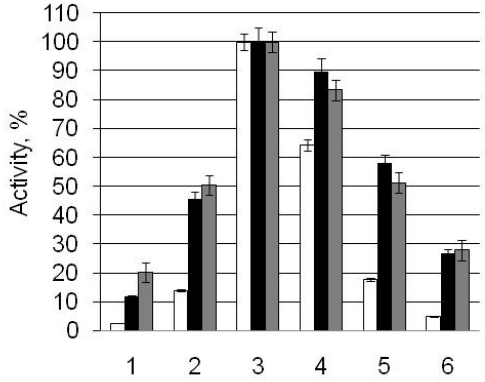

Figure 3. Activities of soluble (□) and covalently immobilized on CHS-GC (■) and PU (▒) *E. aerogenes* lipase towards p-nitrophenyl esters: 1 - pNA-acetate (C3), 2 - pNP-butyrate (C4), 3 - pNP-caprylate (C8), 4 - pNP-caprate (C10), 5 - pNP-laurate (C12) and 6 - pNP-myristate (C14).

Lipase-catalysed transesterification

Lipase is known not only to catalyze hydrolysis, but also the reverse reaction: esterification and transesterification. In the presence of oils (fatty acid glycerol esters) and alcohol, new fatty acid ester is produced and glycerol is released. Enzymatic synthesis of methyl fatty acid esters is a promising field in biotechnological applications as means for environmental-friendly biodiesel synthesis.

The performance of lipase adsorbed on magnetic chitin was studied in the synthesis of methyl-esters. The reaction products were analyzed by TLC after 1, 3, 24, 72 and hours. The greatest yields of methyl ester, monoolein and diolein were reached after 72 hours, whereas the concentration of rapeseed oil in reaction mixture obviously decreased. The existence of methyl ester was detectable after 3 hours of reaction (Figure 4). After 124 hrs of reaction quantitative evaluation of reaction products has shown 21% oleic acid, 35% methyl oleate, 44% glycerol mono-, di- and tri- esters yield.

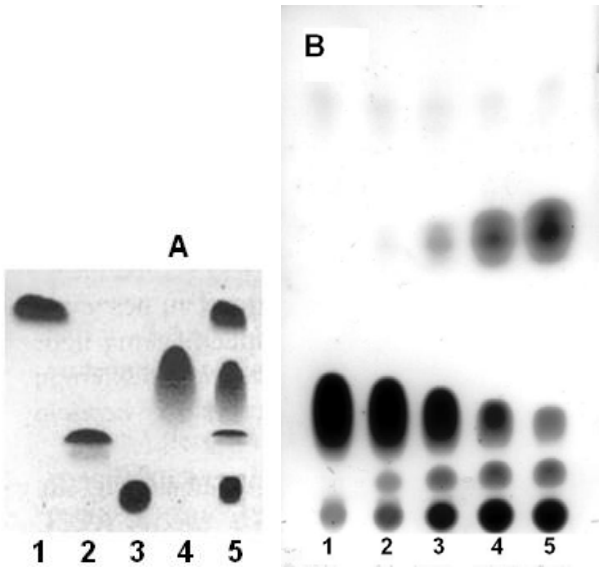

Figure 4. Thin - layer chromatography of lipase – catalyzed transesterification of rapeseed oil with methanol. (A reaction mixture containing 50 mM Tris-HCl buffer (pH 8.0), rape seed oil, methanol and lipase absorbed on magnetic chitin derivative was incubated for an 72 hours at 20°C. A - Control: 1 – TO; 2 – DO (1,3-DO-85%, 1,2-DO-15%); 3 – 1-MO; 4 – oleic acid; 5– mixture of al components (ratio 1:1:1:1). B - Lanes: 1 - rapeseed oil (control), 2-5 - products of reaction after 1, 3, 24, 72 h (respectively).

Immobilization of Enterobacter aerogenes 13 lipase on solid supports

The immobilization efficiency dependence on the support nature was studied. Pure and modified chitosan, lignin, polyurethane and Granocell® were the polymers selected to immobilize lipase by adsorption and covalent attachment. The highest activities were obtained for preparations of covalently immobilized lipase on Granocell 2000® ir Granocell 4000® (36 and 38 Units·g^{-1} preparation respectively). Immobilized protein yield was 31-39% in case of PU support, 38.5% in case of chitosan (CHZ) support and 51-53% in case of Granocell® supports (Table 1).

Table 1. Covalent (GA activated) immobilization of *Enterobacter aerogenes13* lipase on different solid supports: pure polyurethane (PU); macroporous cellulose Granocell - 2000 (Gr-2000) and Granocell - 4000 (Gr-4000); chitosan and poly(ethylene glycol) methyl ether acrylate graft copolymer (CHS-GC)

Crude lipase sample No.	Solid supports	Amount of protein used for immobilization, (mg·g^{-1})	Covalently immobilized enzyme	
			Protein loading efficiency (% of total enzyme added)	Units·g^{-1} of preparation
1	Gr-2000	37,02	51,8	36,227
1	Gr-4000	37,02	52,7	38,842
2	PU	16,22	31,3	3,072
2	PU	24,33	34,8	12,273
2	PU	40,54	34,3	20,861
2	PU	81,10	38,6	30,006
2	CHS-GC	81,10	38,5	3,552

Conclusions

Lipase *Enterobacter aerogenes* 13 immobilized on PU microparticles has higher thermostability in comparison to its soluble form. The temperature optimum of catalytic action of the immobilized enzyme was found to be shifted up to 45°C and pH optimum was lower to compare to native one.

Immobilized enzyme was success fully used for hydrolysis of p-nitophenyl esters of fatty acids with various chain lengths. It was noticed that relative catalytic activities of immobilized enzymes were significantly higher than that of soluble in case of all other esters. Therefore immobilization broadens the enzyme specificity range.

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