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Aspergillus niger lipases: induction, isolation and characterization of two lipases from a MZKI A116 strain

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Abstract

Aspergillus niger strain MZKI A116 was used to produce lipolytic enzymes in submerged culture. Lipase production was induced by addition of olive oil to a complex medium with an initial pH of 5.0. Maximal activity was reached after 70 h in a 15 l bioreactor at 30°C with aeration of 0.5 vvm and agitation 400 rpm. Optimal temperature and pH conditions for the action of the lipases tested on tributyrin were 45°C and pH 7.0. Triacetin and tributyrin were shown to be the best substrates. The presence of iron and silver ions at low concentrations did not alter the activities. Two enzymes possessing lipase activity were isolated by acetone precipitation followed by ion-exchange chromatography. The molecular weights were 43 kDa and 65 kDa with isoelectric points of pH 4.1 and 4.2, respectively. The higher molecular weight lipase showed preference toward 1- and 3-positions of the triglyceride molecule and was stereoselective for the *sn*-1 position with an enantiomeric excess of 20%. It displayed strong activity toward naphthyl, indolyl, umbelliferyl and resorufin esters and was active on esters of hydroxynaphthoic acid anilide, while it showed no activity toward esters of hydroxypyrene trisulfonic acid.

Keywords: Lipases; Aspergillus niger; Selectivity

1. Introduction

Several *Aspergillus niger* strains have been shown to produce lipases (EC 3.1.1.3). Some of these have been well characterized, the N-terminal sequences of two have been determined [1,2] and the crystallization of only one has been described [3]. Various cultivation conditions were employed for the production of lipases. Solid [4] or 'semi-solid' [3,5] cultures were favourable for some strains, while submerged cultivation was preferable for others [6]. Lipase synthesis and secretion required induction by some strains.

Most of the studies on their application have used commercially available lipases as crude preparations or partially purified enzymes. These enzymes were shown to be highly specific catalysts suitable for asymmetric hydrolysis to obtain optically active esters or alcohols [7–9]. In addition, several lipases were used as catalysts for production of aliphatic, aromatic and other esters by direct synthesis [10–12] or transesterification [13,14] in non-aqueous [15] and biphasic systems. Their use in regioselective deacylation of carbohydrates was also described [16,17].

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It was shown that *Aspergillus niger* lipases can be used for enantioselective hydrolysis of some hydrophobic racemic amino acid derivatives as with proteases and acylases [18,19].

Differing results were obtained when enzymes from the same microbial species [20] were compared, suggesting the existence of several different *Aspergillus niger* enzymes. This could be attributed to variations within the *Aspergillus niger* group and the conditions employed for their cultivation.

This study was undertaken to produce new *Aspergillus niger* lipases and to compare their properties to those of the other lipases from the same species. It is part of on-going research aimed at the development of specific fungal lipases.

2. Experimental

2.1. Materials and methods

2.1.1. Organism

Aspergillus niger strain A116 from the MZKI Culture Collection, Ljubljana, was maintained by periodic transfers on beer wort slants. It was grown for seven days at 30°C and then stored at 4°C until further use.

2.1.2. Production

A 15 l Bioengineering L 1523 fermenter (Bioengineering AG, Wald, CH) containing 10 l of medium composed of, olive oil 2% as an inducer, starch 0.1%, KH_2PO_4 0.2%, NH_4NO_3 0.1%, and $MgSO_4 \cdot 7H_2O$ 0.1%, pH of 5.0, was inoculated with a suspension of spores (5×10^8 spores/50 ml of sterile water). The fermentation was conducted at 30°C, agitation 400 rpm and at an aeration of 0.5 vvm.

2.1.3. Isolation and purification

The biomass was filtered off and the fermentation broth treated with 3 vol. (v/v) of cold acetone added dropwise while stirring at 4°C. The precipitate was collected after centrifugation (9000 rpm/15 min), dissolved in 25 mM Tris–HCl buffer, pH 7.4, and freeze-dried. The crude enzyme preparation obtained in this way was dissolved in 25 mM Tris–HCl buffer, pH 7.4, and subjected to ion-exchange chromatography on a Mono Q column (Pharmacia HR 5/5) using the FPLC system. The enzymes were eluted by a gradient of 25 mM Tris–HCl (pH 7.4) and 0–500 mM NaCl in the same buffer at a flow rate of 1 ml/min.

2.1.4. Assays for lipase activity

Three different assays were used:

(i) pH-stat method: using tributyrin as the substrate [21].

(ii) Extraction method: was performed with various simple triglycerides (see Section 2.1.9., *Fatty acid selectivity*). The assay mixture contained 200 mM triglyceride, 0.4 g gum arabic, 100 mM $CaCl_2$ solution 1 ml, 100 mM Na-phosphate buffer, pH 7.0, 4 ml and lipase solution 1 ml; and was incubated at 40°C for 2 h with constant agitation (200 rpm) in conical flasks. The reaction was stopped by addition of 1 N H_2SO_4 (1 ml) and the released fatty acids extracted with petroleum ether (40–70°C) and titrated with 0.01 M KOH to pH 7.0.

(iii) Activity staining: was performed on renatured (Na–phosphate buffer) SDS polyacrylamide gels, native gels and isoelectric focusing gels. The chromogenic substrates were α- and β-naphthyl esters (acetate, propionate, butyrate and valerate), naphthyl AS acetate, naphthyl AS-2-chloropropionate and naphthyl AS-BI butyrate. The gels were placed in a solution containing 1 ml of substrate (60 mg in 5 ml acetone), 0.1 M Na–phosphate buffer, pH 7.0, 10 ml and 250 μl dye solution (100 mg Fast Blue RR salt in 5 ml water) and were incubated until coloured bands could be visualized. When fluorescent substrates were used (4-methylumbelliferyl butyrate, resorufin butyrate, 5-(and 6-)-carboxyl-2′,7′-dichlorofluorescein diacetate, 1-acetoxypyrene-3,6,8-trisulfonic acid and 1-butyroxypyrene-3,6,8-trisulfonic acid) the gels were placed in a solution containing 10 μl

substrate (1 mg/ml dimethyl sulfoxide) and 0.1 M Na–phosphate buffer, pH 7.0, 1 ml. The appearance of fluorescent bands was observed under UV light. [1]

2.1.5. Electrophoretic methods

The molecular weight was determined by SDS–PAGE (Bio-Rad Mini Protean) on 12% polyacrylamide gels [22]. The proteins were detected by silver staining. Samples which were stained for activity were prepared similarly without the 95°C denaturation step [22]. After electrophoresis the gels were renatured by washing out the SDS for 30 min with 100 mM Na–phosphate buffer.

Native polyacrylamide gels were made without SDS [23] by using a 5% stacking gel in 0.062 M Tris buffer, pH 6.8, and 12% separating gel in 0.33 M Tris buffer, pH 7.8. The running buffer was composed of 0.049 M Tris and 0.038 M glycine (pH 8.0). Separation was conducted at 150 V at room temperature.

The isoelectric points were measured by isoelectric focusing (Pharmacia Multiphor II system) using Ampholyne PAG plates with pH 3.5–9.5. Prefocusing and focusing were performed at constant current (8 mA/20 min, 14 mA/2.5 h) and temperature (10°C). The proteins were stained with Coomassie blue R-250 as outlined in the manufacturers manual or for activity.

2.1.6. Protein determination

The protein content during fermentation and purification steps was assayed by the BCA (bicinchoninic acid) method [24], available as a kit from Pierce (Rockford, IL). Bovine serum albumin was used as a standard.

[1] Naphthol AS = 3-hydroxy-2-naphthoic acid anilide; naphthol AS-BI = 6-bromo-2-hydroxy-3-naphthoic acid 2-methoxyanilide; SDS = sodium dodecyl sulphate; PAGE = polyacrylamide gel electrophoresis.

2.1.7. Optimum pH and temperature for lipase activity

Lipase activity was followed by the pH-stat method at pH values in the range pH 5–11, and temperatures in the range 35–75°C, to determine the optimal pH and temperature conditions.

2.1.8. Influence of effectors on lipase activity

The activity was measured after addition of $AgNO_3$, $CaCl_2$, $FeCl_3$, $MgCl_2$, NaCl, and SDS to the reaction mixture in concentrations from 1 mM to 100 mM and compared to that without an effector.

2.1.9. Fatty acid selectivity

Activity toward different monoacid triglycerides of various acyl chain lengths was measured by the extraction method. The triglycerides tested were: triacetin (C2), tributyrin (C4), tricaprylin (C8), tricaprin (C10), trilaurin (C12), trimyristin (C14), and triolein (C18).

2.1.10. Regioselectivity

Regioselectivity was tested with triolein at various periods of lipolysis according to Stadler et al. [25]. The reaction products were separated by TLC. The ratio between the formed products was calculated from the integrated peak areas obtained by scanning the plates at 400 nm.

2.1.11. Stereoselectivity

Stereoselectivity was determined toward triolein [25]. Diglyceride mixtures obtained by lipolysis were derivatized with optically active *R*-(+)-phenylethylisocyanate in the presence of 1,4-diazabicyclo[2.2.2]octane. The crude urethane derivates were purified by TLC and the phenylethylurethane derivatives extracted from the silica gel. The carbamate mixtures were resolved by HPLC (Bio-Rad HPLC-gradient module 800 equipped with a 300 × 4 mm silica gel column, Eurospher 100, 6 μm) and the ratio of hydrolysis in positions *sn*-1 and *sn*-3 calcu-

lated after integration of the corresponding HPLC areas.

3. Results and discussion

Olive oil was needed for the induction of lipases by our strain. The activity was detected after 40 h of fermentation and increased steeply, reaching a maximum at 70–90 h (Fig. 1.). In previous work [26] we observed a similar level of lipase production when soybean, sunflower or rapeseed oil were used instead of olive oil. Without the addition of lipid materials, however, lipase activity was hardly detected in the broth. An essential role of lipid materials in lipase synthesis was also reported by Pal et al. [6] who observed significant differences in lipase secretion with various lipids in the cultivation medium, olive oil being the best inducer. Birnescu and Vasu [5] obtained the highest production of lipases on a medium containing mainly sun flower groats and starch. The strain studied by Fukumoto et al. [3] did not require an inducer and produced lipases in a medium with $CaCO_3$.

Organic solvents were employed for the concentration of the cell-free culture broth and dissociation of the bonded lipids remained in the filtered broth. Acetone and iso-propanol were used at different concentrations. At lower concentrations (< 2 v/v) higher activities were observed with iso-propanol while at higher concentrations (> 3 v/v) acetone resulted in a 3-fold increase in total lipase activity per mg protein.

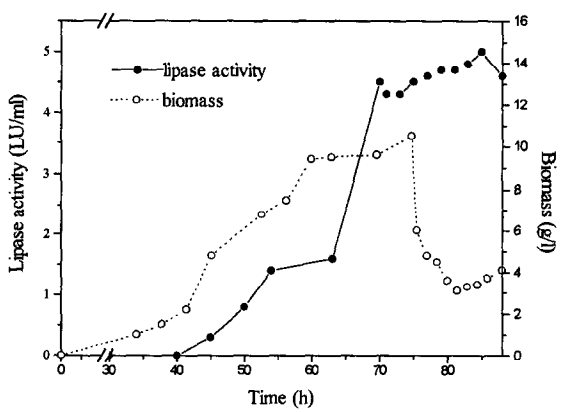


Fig. 1. Time course of lipase production and biomass.

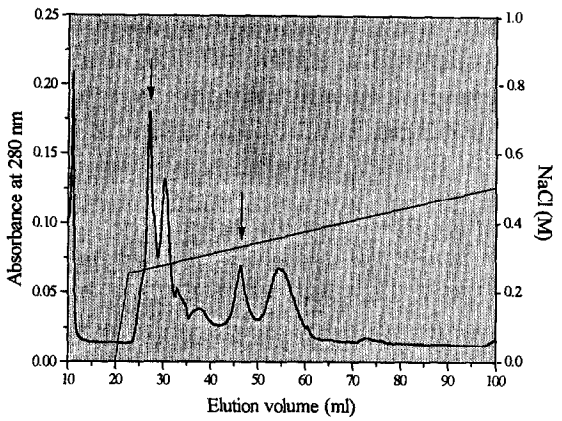


Fig. 2. Elution profile of Mono Q ion-exchange chromatography. The lipase peaks are indicated by arrows.

The separation of lipases was performed with Mono Q ion-exchange chromatography of the precipitated crude enzyme preparation. Lipase activity eluted in one sharp peak at about 300 mM NaCl followed by a broader peak (Fig. 2.). The active fractions were collected and analyzed by polyacrylamide gel electrophoresis. Denaturing and non-denaturing conditions were used in the presence or absence of SDS. The first fraction pool contained a 65 kDa lipase and second a 43 kDa lipase (Fig. 3.). The specific activity of the higher molecular weight lipase was 250 LU/mg.

Two active peaks upon elution of *Aspergillus niger* lipases from a Mono Q column were also observed by Torossian and Bell [2] from a commercial preparation. Their second peak exhibited one major 37 kDa and two minor polypeptide bands.

A commercial *Aspergillus niger* lipase preparation investigated by Tombs and Blake [1] gave a 48 kDa band by SDS-PAGE. A decrease in the molecular weight was observed upon boiling the enzyme or when four mannose residues were removed, resulting in a 34 kDa

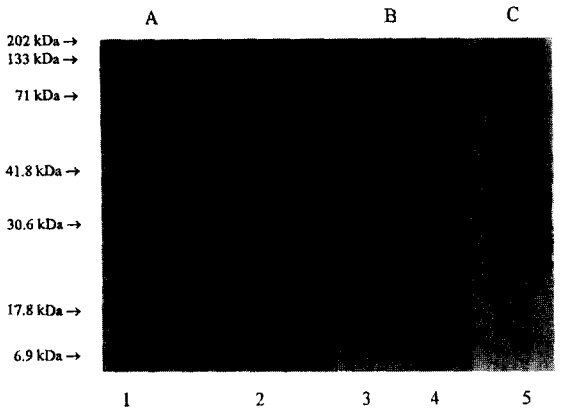


Fig. 3. Polyacrylamide gel electrophoresis of *A. niger* lipases and standard proteins. Gels (A) and (B), SDS–PAGE renatured and stained for activity, gel (C), PAGE stained for activity. Lanes 1 and 4, kaleidoscope molecular weight standards (Bio-Rad): myosin (202 kDa), β-galactosidase (133 kDa), bovine serum albumin (71 kDa), carbonic anhydrase (41.8 kDa), soybean trypsin inhibitor (30.6 kDa) and lysozyme (17.8 kDa), aprotin (6.9 kDa). Lane 2, the 43 kDa lipase, and lane 3, the 65 kDa lipase. Lane 5, the 43 kDa and the 65 kDa lipase.

band. A similar value (35 kDa) was also reported for another preparation by Sugihara et al. [27], while Höfelmann et al. [28] reported on two enzymes of 31 kDa and 19 kDa, respectively.

The two lipases showed isoelectric points of p*I* 4.1 and 4.2 when analyzed by isoelectric focusing (Fig. 4.). These values are in the range reported for *Aspergillus niger* lipases discussed above (p*I* 3.5–4.5).

Enzyme activity was detected over the range of pH 5–11, the optimum being pH 7.0, whereas optimal acidic pH values of 4.5–6 were reported for other strains [3,1,27,28]. The optimal temperature was 40°C, a sharp decrease in activity was noticed at temperatures above 55°C. Lower optimal temperatures (25°C) were reported by Sugihara et al. [27] and Fukumoto et al. [3].

The influence of effectors on the activity are presented in Fig. 5. All compounds had a stimulating effect at 1 mM concentration with the exception of SDS which caused a 57% decrease in activity. The presence of $FeCl_3$ caused a significant decrease in activity at 10 mM con-

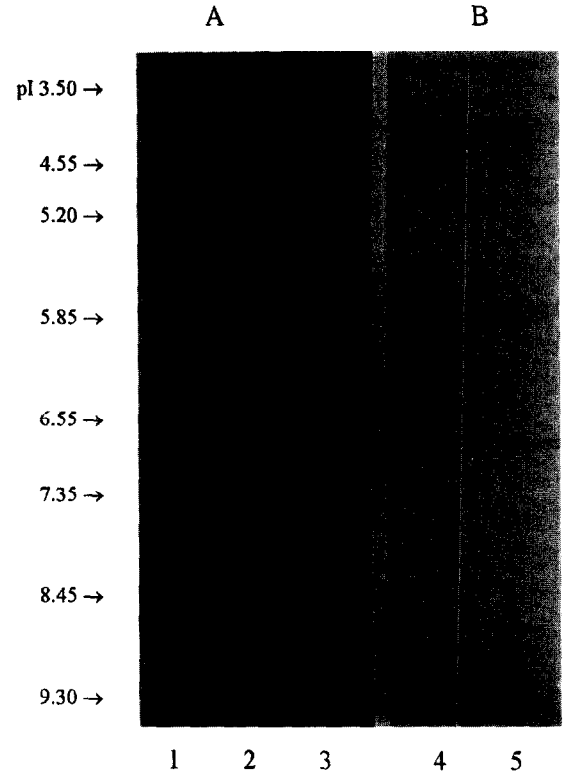


Fig. 4. Isoelectric focusing of *A. niger* lipases and standard proteins. Gel (A) stained with Coomassie blue R-250, gel (B) stained for activity. Lane 3, standard proteins (Pharmacia): amyloglucosidase (p*I* 3.5), soybean trypsin inhibitor (p*I* 4.55), β-lactoglobulin A (p*I* 5.2), bovine carbonic anhydrase B (p*I* 5.85), human carbonic anhydrase B (p*I* 6.55), myoglobin acidic band (p*I* 6.85), myoglobin basic band (p*I* 7.35), lentil lectin acidic band (p*I* 8.15), lentil lectin middle band (p*I* 8.45), lentil lectin basic band (p*I* 8.65), trypsinogen (pI 9.3). Lanes 1 and 4, the 65 kDa lipase. Lanes 2 and 5, the 43 kDa lipase.

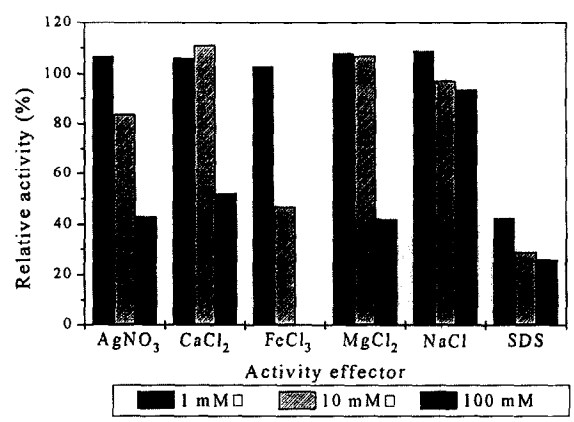


Fig. 5. Influence of effectors on lipase activity. Relative activity is expressed as % of that with no effector (100%).

Table 1
Activity of the 65 kDa lipase on various chromogenic and fluorescent substrates tested by staining in native polyacrylamide gels

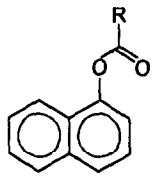
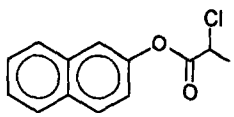
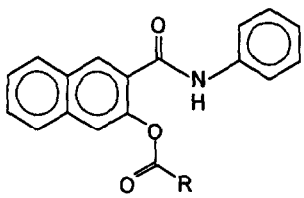
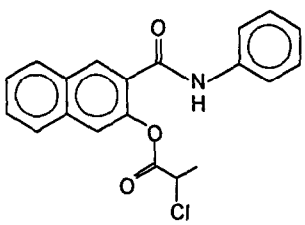
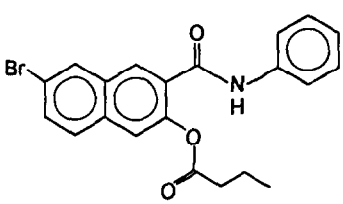
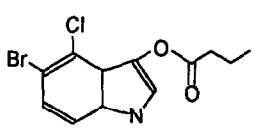
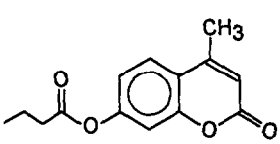
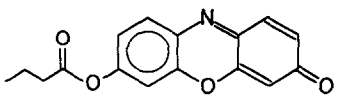
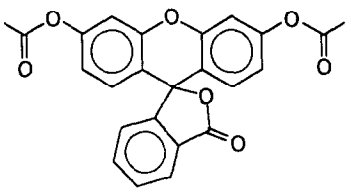
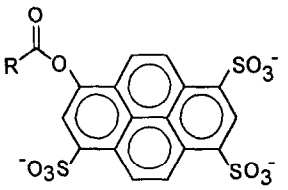
Substrate	Structure	Activity
α- and β-naphthyl-acetate α- and β-naphthyl-propionate α- and β-naphthyl-butyrate α- and β-napthyl-valerate		+ + + + + + + +
β-naphthyl-2-chloropropionate		+ +
naphthyl AS-acetate		+
naphthyl AS-2-chloropropionate		+
naphthyl AS-BI-butyrate		+
5-bromo-4-chloro-3-indolylbutyrate		+ +
4-methylumbelliferylbutyrate		+ +

Table 1 (continued)

Substrate	Structure	Activity
resorufin-butyrate		+ +
5-(and 6-)carboxyl-2',7'-dichlorofluorescein diacetate		+
1-acetoxypyrene-3,6,8-trisulfonic acid 1-butyroxypyrene-3,6,8-trisulfonic acid		– –

+ + strong activity; + active; – no activity

centration, while it was completely inhibitory at 100 mM concentration. Other enzymes were reported to be severely inhibited by iron ions present in tap water used in the dialysis for the purification of the enzyme [29]. About 90% of activity was lost in the presence of 1 mM iron ions and 8 ppm iron ions (Fe^{2+}) in the reaction mixture [27,29]. A stronger inhibition by $AgNO_3$ at lower concentrations compared to our enzymes was also reported [28].

The 65 kDa lipase was tested for activity toward various chromogenic and fluorescent substrates. The activity was judged by comparing the intensity of coloration of the bands in the polyacrylamide gels. The results summarized in Table 1 show that there is strong activity toward 1- and 2-naphthyl esters in either α- or β-configurations, 3-indolyl ester, 4-methylumbelliferyl ester and resorufin ester. A lower activity was observed on the naphthyl ester with a 2-naphthoic acid anilide. The presence of chlorine and bromine atoms on the naphthyl or indolyl moiety did not influence the activity of the enzyme. No activity was detected when 1-hydroxypyrene-3,6,8-trisulfonic acids were used as substrates.

The activity on monoacid triglycerides having various acyl chain lengths is presented in Table 2. Higher activity was displayed toward triglycerides having short chain lengths (C2–C4), while preference toward medium chain length triglycerides (C8–C12) has been described [30].

Previous studies with *Aspergillus niger* lipases have indicated that they display regioselectivity toward 1- and 3-position of the glycerol moiety [31]. Triolein was hydrolysed by the 65 kDa lipase to 1,2(2,3)-diolein, monoolein and free fatty acids (Table 2). Small quantities of 2,3-diolein were also detected and we suppose their presence is due to spontaneous isomerization. Our results supplement the previously reported findings on the positional specificity. Stereoselectivity tests on triolein revealed selectivity toward the *sn*-1 position (Table 2).

The lipases investigated in this study differ from other *Aspergillus niger* lipases with respect to molecular weight, pH and temperature

Table 2
Fatty acid selectivity, regioselectivity and stereoselectivity of the 65 kDa lipase

Fatty acid selectivity [a]	short-chain triglycerides
triacetin (C-2)	100%
tributyrin (C-4)	92%
tricaprylin (C-8)	26%
tricaprin (C-10)	20%
trilaurin (C-12)	33%
trimyristin (C-14)	28%
triolein (C-18)	26%
Regioselectivity [b]	1,3
1,2(2,3)-diolein	49%
1,3-diolein	4%
monoolein	8%
oleic acid	39%
Stereoselectivity [b]	*sn*-1
2,3-DG-carbamate	60%
1,2-DG-carbamate	40%
Enantiomeric excess	20%

[a] Values expressed as % of that on triacetin (100%).
[b] Determined on triolein.

optima, stability toward iron and silver ions, and fatty acid selectivity. Possible application of these enzymes for specific reactions are under study.

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