Purification and some characteristics of *Penicillium citrinum* lipase

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1. Introduction

Lipase [EC 3.1.1.3] is characterized by its ability to catalyze the hydrolysis of triacylglycerols at the interface between oil and water (1-3). It is well known that the reaction is reversible and that the enzyme can catalyze ester synthesis as well as transesterification in reaction systems containing low concentration of water. Furthemore, lipase can act even in nearly anhydrous organic solvents and it catalyzes stereoselective and regioselective reactions (4-5). Lipases differ from each other in their physical and biochemical properties such as optimal reaction conditions, molecular weights and substrate specificity. Consequently, these enzymes have potential applications in various industries (6-7).

Filamentous fungi are preferred sources of lipases because they produce extracellular enzymes.

The most productive species belong to the genera *Rhizopus*, *Rhizomucor*, *Mucor*, *Geotrichum*, *Aspergillus* and *Penicillium* (8-9).

As previously reported, we isolated a filamentous fungus *Penicillium citrinum*, producing extracellular lipase (10), determined the optimal culture conditions for the production of the lipase (11) and described some properties of the crude enzyme (12).

This paper reports on the purification of *Penicillium citrinum* lipase together with some of its biochemical and physicochemical properties.

2. Materials and methods

2.1. Fungal strain

A wild strain of *Penicillium citrinum* was isolated from soil as described previously (10).

2.2. Culture conditions

The microorganism was cultivated in a medium containing (g/l): peptone, 50.0; starch, 20.0; $(NH_4)_2SO_4$, 5.0; $MgSO_4$ x $7H_2O$, 0.25; $CaCO_3$, 5.0; K_2HPO_4, 0.5; yeast extract, 1.0 and 0.7% v/v Tween 80.

The culture was grown in Erlenmeyer flasks at 22°C with shaking. The culture was filtered and the cell free filtrate used as a source of extracellular lipase.

2.3. Determination of lipolytic activity

The reaction mixture consisted of 3 ml tributyrin/ olive oil- polyvinyl alcohol (PVA) emulsion (10% tributyrin or olive oil in 2% PVA), sonicated for 10 min in ice with ultrasonic desintegrator, 2 ml of 0.05 M Tris-HCl buffer (pH 7.0) and 1 ml of the enzyme solution. The mixture was incubated for 1h at 30°C with shaking. Enzyme reaction was terminated by addition of 20 ml of acetone-ethanol mixture (1:1 v/v). Released fatty acids were titrated with 0.05 M NaOH in the presence of thymolphthalein. One unit of lipase activity (A_h) was defined as the amount of enzyme which liberated 1 μmol of free fatty acid per minute under test conditions.

2.4. Determination of protein

The protein concentration in the culture supernatant and chromatography fractions were determined using Peterson's modification of the Lowry's method (13). Bovine serum albumin was used as the standard.

2.5. Purification of lipase

All the purification steps were carried out at 4°C.

Step 1. Ammonium sulfate fractionation

Ammonium sulfate was added to the supernatant to give a concentration of 40% saturation. After stirring for 1 h and centrifugation for 15 min at 10 000 x g, precipitate was discarded and $(NH_4)_2SO_4$ added to the supernatant to a final concentration of 60%. After stirring for 1 h, precipitate was collected by centrifugation and dissolved in 0.05 M Tris-HCl buffer, pH 7.0. The solution was concentrated to 10 ml using an ultrafiltration membrane (YM 10; 10,000-Da cut-off, Amicon, USA), followed by diafiltration with 5 vol. of 0.05 M Tris-HCl buffer, pH 7.0.

Step 2. Chromatography on Octyl-Sepharose Cl-4B

The concentrated solution was loaded onto an Octyl-Sepharose CL-4B (Pharmacia, Sweden) column (0.9 cm x 30 cm) previously equilibrated with 0.01 M phosphate buffer containing 1 M ammonium sulfate, pH 7.0. The column was washed with 10 times the bed volume of the same buffer con-

taining 50 mM ammonium sulfate and then 0.01 M phosphate buffer without $(NH_4)_2SO_4$. Washing with phosphate buffer was carried out until the eluate absorbance at 280 nm fell below 0.01. The column was then washed with 5 times the bed volume with demineralized water. The lipase was eluted by a linear gradient of demineralized water and 1% Triton X-100. The fractions with lipase activity greater than 200 A_h/mg (fractions 75-79) were pooled and dialyzed overnight against 0.01 M phosphate buffer, pH 7.0.

2.6. Disc electrophoresis

Disc electrophoresis was performed according to the method of Davis using 7.5% polyacrylamide gel (14). Protein was stained with Coomasie Blue R-250. Lipase activity was detected using the tributyrin agar plate test (15). The gels from disc electrophoresis were placed on the surface of tributyrin agar and incubated for 24 h at 30°C. Zones of tributyrin hydrolysis were observed after the incubation time.

2.7. Estimation of M_r

In order to determine molecular weight, the enzyme was electrophoresed on 7.5% polyacrylamide gel containing 0.1% SDS, according to the method of Weber and Osborn (16). The following proteins were used as standards: native PAGE: phosphorylase B (M_r 97,400), β-galactosidase (M_r 116,000) and myosin (M_r 205,000); SDS-PAGE: lysozyme (M_r 14,000), β-lactoglobulin (M_r 18,400 in subunits), trypsinogen (M_r 24,000), pepsin (M_r 34,700), ovalbumin (M_r 45,000), bovine serum albumin (M_r 66,000).

2.8. Substrate specificity

The substrate specificity towards different substrates: olive oil, sunflower oil, rape seed oil, triacetin, tributyrin, tricaprin, trilaurin, tripalmitin, triolein, caproic acid methyl ester and stearic acid methyl ester was analyzed by the titration method.

2.9. Thin-layer chromatography

The positional specificity of the lipase was analysed by a modified method described earlier for lipase from *Penicillium simplicissimum* (17). Plates of silica gel 60 F_{254} (E.Merck, Darmstadt, Germany) were used for TLC analysis of lipolysis. Substrates 1-monooleoyl-glycerol, 1,3-diolein, 1,2-dioleoyl-rac-glycerol and triolein in a final concentration of 15 mM were emulsified in 500 µl of 10% polyvinyl alcohol. After addition of lipase, samples were incubated for 2 h at 30°C with shaking. Samples without enzyme were used as a control. The mixture hexan:diethyl ether: acetic acid (80:20:1, v/v) was

used for plate developing. Spots of the hydrolysis products were visualized by spraying with 50% sulphuric acid and heating for 15 min at 150°C.

2.10. The effect of pH on lipase activity

Lipase activity was determined at several pH values (5.0 – 9.0) by the titrametric method described above.

2.11. The effect of temperature on lipase activity

For the determination of the optimum temperature, the reaction mixture was incubated at intervals between 10°C and 50°C.

2.12. Stability to different pH values

Lipase was preincubated at 4°C in buffers of different pH values. After 1 h, enzyme activity was measured at pH 7.2.

2.13. Temperature stability

Lipase was incubated for 1 h at temperatures between 10°C and 40°C and then allowed to cool for 1 h at 4°C before lipase activity was measured at 30°C. Besides, aliquots of the enzyme were stored for 9 months at 4°C. Every 3 months, a sample was carried out and lipase activity (with olive oil as substrate) was measured.

3. Results and discussion

When culture supernatant was salted out with 40% saturated ammonium sulfate, some contaminating proteins were separated from other medium components at high ionic strength. As this floating material did not exhibit lipase activity, it was discarded. Then $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 60%. This floating material had lipase activity. After this precipitation the enzyme exibited a 10,5-fold higher specific activity in comparison to the culture supernatant. Then unnecessary proteins and ammonium sulfate were removed by ultrafiltration. The lipase was purified by Octyl-Sepharose CL-4B column chromatography as shown in Figure 1.

The lipase was purified 400-fold with a specific activity of 560 A_h/mg protein (Table 1). The purified enzyme showed only one protein band on disc-PAGE (Fig. 2a).

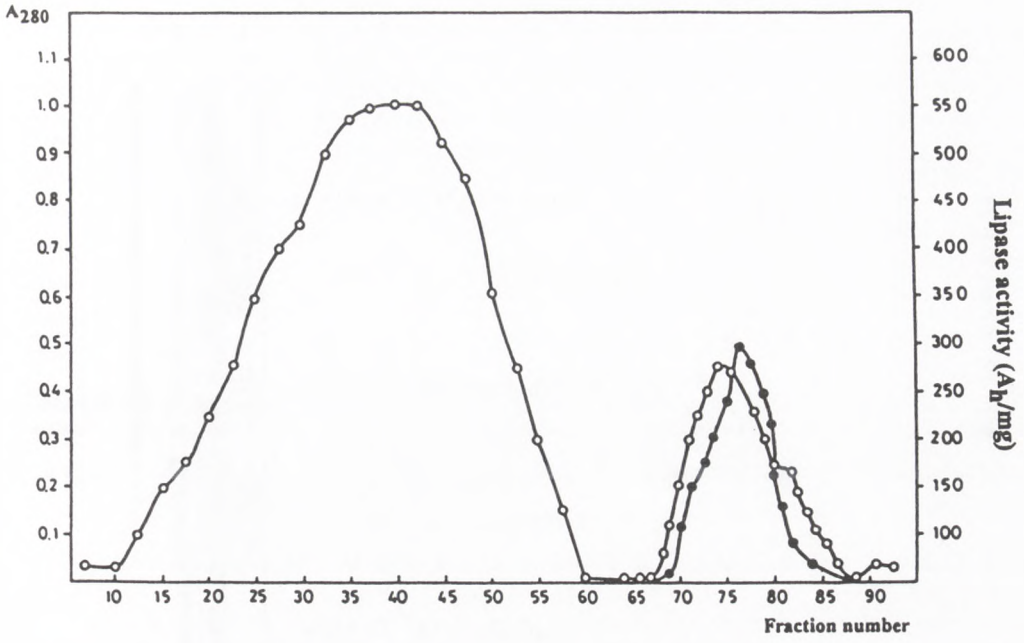

Fig. 1. Elution pattern of lipase from *Penicillium citrinum* on Octyl-Sepharose CL-4B. The lipase was eluted by linear gradient of demineralized water and 1% Triton X-100. (O) A_{280}; (●) lipase activity with olive oil as substrate (A_h/mg).

TABLE 1
SUMMARY OF THE PURIFICATION OF LIPASE FROM *Penicillium citrinum*

Step	Total protein (mg)	Total units activity ($A_h \times 10^3$)	Specific activity (A_h/mg)	Yield (%)	Purification factor
Culture filtrate	7100	9.94	1.4	100	1
$(NH_4)_2SO_4$ precipitation	463.9	6.82	14.7	68.6	10.5
Ultrafiltration concentrate	93.0	5.96	64.1	56.3	45.8
Octyl-Sepharose	1.71	0.96	560	9.66	400

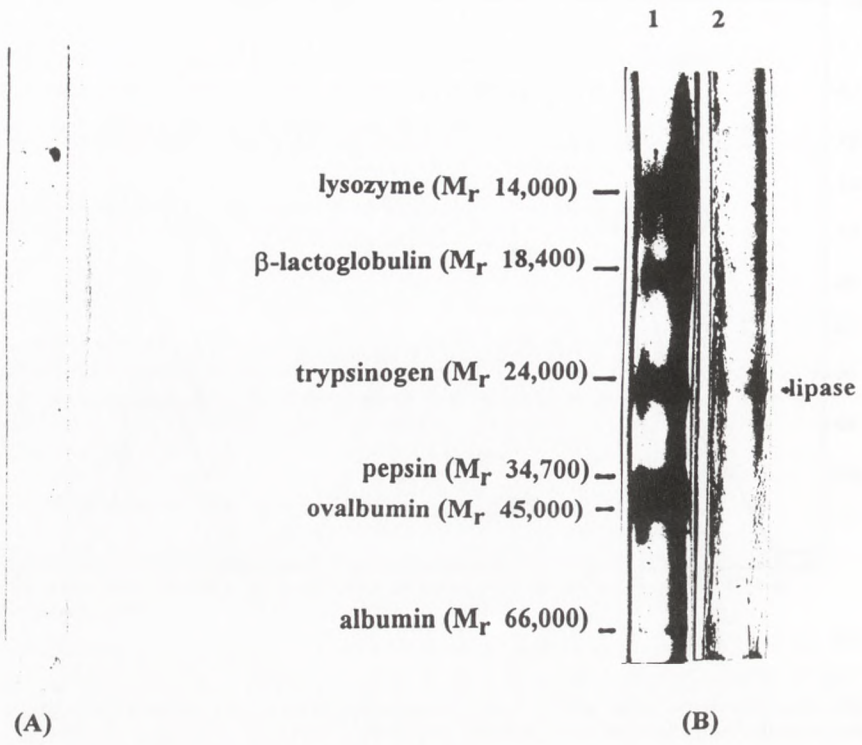

Fig. 2. PAGE and SDS-PAGE electrophoresis of *Penicillium citrinum* lipase in 7.5% polyacrylamide gel. (A) disc-PAGE; SDS-PAGE; line 1-molecular markers; 2-lipase.

A molecular mass for the purified enzyme was determined by SDS-PAGE (Fig. 2b). The figure indicated the presence of a single band having a molecular weight of 26 000. During native PAGE the purified lipase migrated as a dimer and tetramer with molecular weight of 52 kDa and 108 kDa, respectively.

It was impossible to determine molecular weight by gel filtration because the lipase formed various aggreggates. This effect was also observed in the case of *Penicillium simplicissimum* (17) and *Penicillium expansum* lipase (18).

As shown in Table 2, the lipase from *Penicillium citrinum* was highly specific for acylglycerols, whereas fatty acid esters were hydrolyzed at a rate of only 5–6%. Tributyrin was the best substrate for purified *P. citrinum* lipase. These observations generally agree with those reported for other *Penicillia* lipases (19-21). Among natural oils tested (Table 2a), the enzyme showed the highest hydrolytic activity towards olive oil.

TABLE 2
RELATIVE ACTIVITY OF *Penicillium citrinum* LIPASE TOWARD VARIOUS SYNTHETIC OILS AND FATTY ACIDS METHYL ESTERS

Substrate	Concentration	Relative hydrolysis (%)*
Triacetin	5 mM	23
Tributyrin	5 mM	100
Tricaprin	5 mM	15
Trilaurin	5 mM	18
Tripalmitin	5 mM	12
Tristearin	5 mM	15
Triolein	5 mM	60
Caproic acid methyl ester	5 mM	6
Palmitic acid methyl ester	5 mM	5

* 100% = lipolytic activity of *Penicillium citrinum* lipase toward tributyrin (5 mM)

TABLE 2a
RELATIVE ACTIVITY OF *Penicillium citrinum* LIPASE TOWARD NATURAL OILS

Substrate	Concentration	Relative hydrolysis (%)**
Olive oil	10% (v/v)	100
Sunflower oil	10% (v/v)	95
Soybean oil	10% (v/v)	30
Rape seed oil	10% (v/v)	62
Corn oil	10% (v/v)	26

** 100% = lipolytic activity of *Penicillium citrinum* lipase toward olive oil (10% (v/v))

The hydrolysis of 1-mono, 1,2-di-, 1,3-di- and triolein by the *Penicillium citrinum* lipase was studied by TLC. All substrates (Fig. 3) were hydrolyzed. So, the lipase from *P. citrinum* is non-specific and hydrolyses the 1-, 2-, and 3-positions of triolein. This is in agreement with the data for *P. simplicissimum* (17) and *P. expansum* lipases (18). Lipase B from *Penicillium camembertii* showed a high preference for monoolein (22). *Penicillium cyclopium* produces two kinds of lipolytic enzymes, one revealed a high preference for mono- and diacylglycerols and the other for triacylglycerols (23). The lipase from *P. citrinum* was also able to hydrolyse p-nitrophenyl palmitate (data not shown).

As reported previously (12), the crude enzyme from *Penicillium citrinum* was stable between pH 5.0 and 7.5 and in the temperature range from 10°C

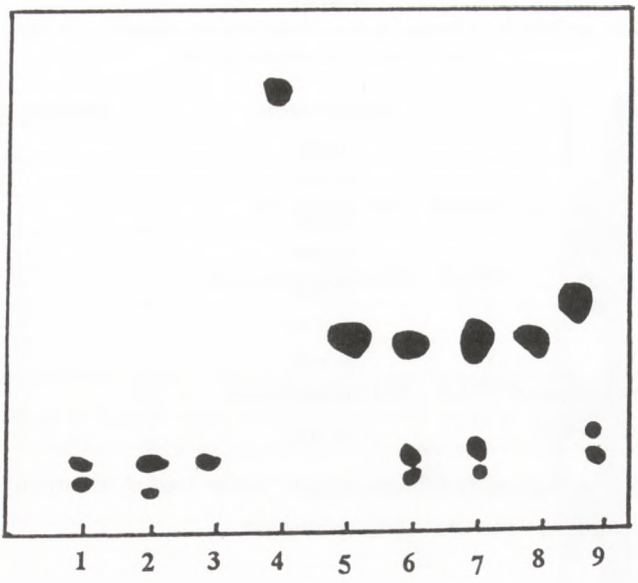

Fig. 3. Thin-layer chromatography of mono-, di- and trioleins hydrolytic products with *Penicillium citrinum* lipase. **1**, 1,2-diolein control; **2**, 1,3-diolein control; **3**, 1-monoolein control; **4**, triolein control; **5**, oleic acid; **6**, hydrolytic products of 1,2-diolein; **7**, hydrolytic products of 1,3-diolein; **8**, hydrolytic products of monoolein; **9**, hydrolytic products of triolein.

to 40°C. Figure 4 illustrates the results of the pH optimization experiment. At pH 7.2 the hydrolysis of both tributyrin and olive oil proceeded at its fastest rate. These results are at variance with those found by Pimentel et. al. (24). The purified lipase was stable between pH 6.0 – 7.5. At pH 8.0 it retained 80% of maximum activity when olive oil was served as substrate and 72% of maximum activity with tributyrin as substrate (Fig. 4).

The results of the temperature optimization test are shown in Figure 5. With either of the two substrates the optimum enzymatic activity for purified enzyme was 25°C – 30°C.

The lipase from *Penicillium citrinum* although not thermostable, even at 40°C, retained 93% of maximum activity with olive oil as substrate and 91% of maximum activity with tributyrin (Fig. 5). The result obtained for olive oil is in agreement with that of Pimentel et al. (24).

The lipase could be stored at 4°C for at least 9 months without any loss of activity in 0.05 mM Tris-HCl buffer (pH 7.2) (Table 3).

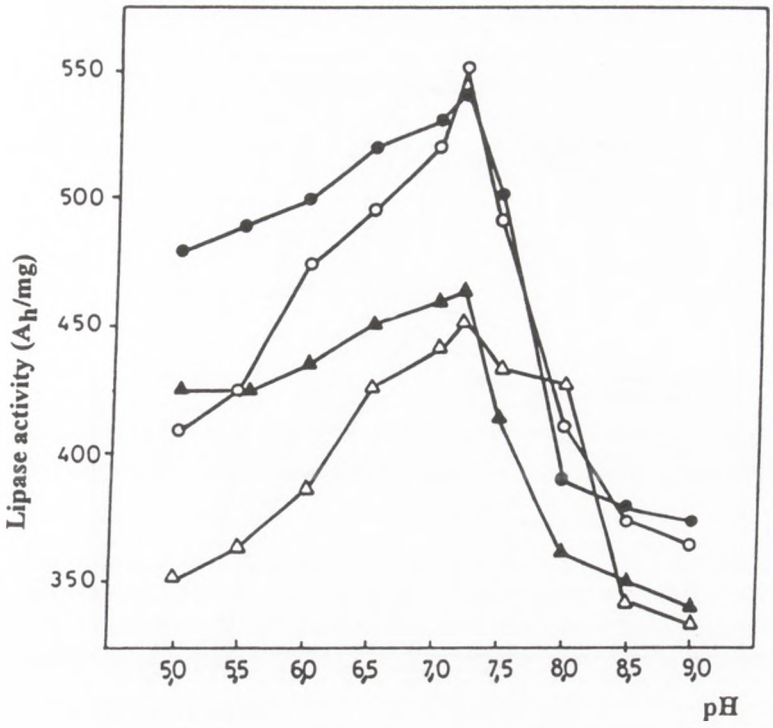

Fig. 4. Effects of pH on lipase activity and stability. (O) lipase activity with tributyrin as substrate; (●) lipase stability with tributyrin as substrate; (Δ) lipase activity with olive oil as substrate; (▲) lipase stability with olive oil as substrate.

TABLE 3
THE CHANGE OF LIPOLYTIC ACTIVITY DURING STORAGE AT 4°C

Time (month)	Lipase activity (A_h/mg)
0	560
3	555
6	555
9	553

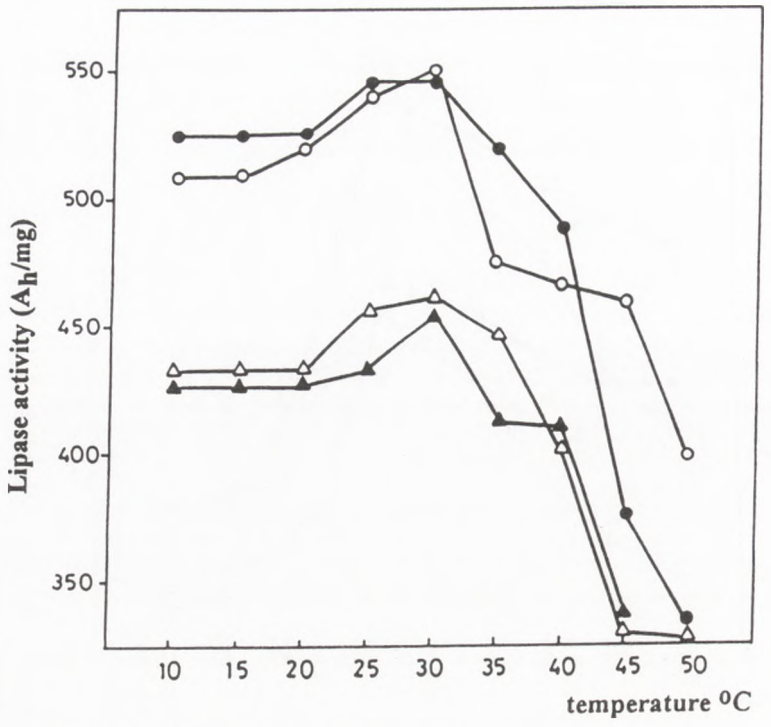

Fig. 5. Effects of temperature on lipase activity and stability. (O) lipase activity with tributyrin as substrate; (●) lipase stability with tributyrin as substrate; (Δ) lipase activity with olive oil as substrate; (▲) lipase stability with olive oil as substrate.

The comparison of properties of lipase from other *Penicillium* species with respect to *Penicillium citrinum* was summarized in Table 4.

TABLE 4
COMPARISON OF SOME PROPERTIES OF *Penicillium* sp. LIPASES

Strain	M_r	Optimum pH	Optimum temp. (°C)	pH stability	Thermal stability	Ref.
P. citrinum	26,000	7.2	30	6.0-7.5	10-40	
P. crustosum						
lipase I	29,300	9.0	—	6.0-9.0	45	(19)
lipase II	32,200	9.0	—	7.0-9.0	45	
P. cyclopium Westring						
lipase A	27,000	7.0	35	6.5-9.0	30	(25)
lipase B	36,000	6.0	40	4.0-6.0	30	
P. cyclopium M_1						
lipase I	54,000	6.0	40	4.0-6.0	40	(26)
lipase II	32,000	6.0	40	4.0-6.0	40	(23)
P. caseicolum	—	9.0	35	—	25	(20)
P. simplicissimum	56,000	5.0	37	5.0-7.0	50	(17)
P. expansum	25,000	9.0	45	6.0-10.0	30	(18)
P. citrinum var. (data for crude enzyme)	—	8.0	34-37	5.0-7.0	45	(24)

References

1. Brockerhoff H., Jensen, R. G., (1974), in: *Lipolytic Enzymes*, Eds. Brockman H., Jensen R. G., Academic Press, New York, 10-23.
2. Brockman H. L., (1981), in: *Lipases*, Eds. Borgstrom B., Brockman H. L., Elsevier, Amsterdam, 3-46.
3. Brzozowski A. M., Derewenda U., Derewenda Z. S., Dodson G. G., Lawson D. M., Turkenburg J. P., Bjorkling F., Huge-Jensen B., Patkar S. A., Thim L., (1991), Nature, 351, 491-494.
4. Goldberg M., Thomas D., Legoy M. D., (1990), Eur. J. Biochem., 190, 603-609.
5. Burgess K., Jenning D. L., (1991), J. Am. Chem. Soc., 16, 6129-6139.
6. Macrae A. R., Hammond R. C., (1985), Biotechnol. Gen. Eng. Rev., 3, 193-217.
7. Harwood J., (1989), Trends Biochem. Sci., 14, 125-126.
8. Iwai M., Tsujisaka Y., (1984), in: *Lipases*, Eds. Borgstroem B., Brockman H. L., Elsevier, New York, 443-469.
9. Godtfredsen S. E., (1990), in: *Microbial Enzymes and Biotechnology*, Eds. Fogarty W. M., Kelly C. T., Elsevier, London, 255-274.
10. Sztajer H., Maliszewska I., Wieczorek J., (1988), Enzyme Microb. Technol., 10, 492-497.
11. Sztajer H., Maliszewska I., (1989), Biotechnol. Lett., 12, 895-898.
12. Maliszewska I., Mastalerz P., (1992), Enzyme Microb. Technol., 14, 190-193.
13. Peterson G. L., (1977), Anal. Biochem., 83, 346-356.
14. Davis B. J., (1964), Ann. of the New York Acad. Sci., 121, 404-427.
15. Arima K., Liu W. H., Beppu T., (1972), Agric. Biol. Chem., 5, 893-895.
16. Weber K., Osborn M., (1969), J. Biol. Chem., 244, 4406-4412.

17. Sztajer H., Lunsdorf H., Erdman H., Menge U., Schid R., (1992), Biochim. Biophys. Acta, 1124, 253-261.
18. Stocklein W., Sztajer H., Menge U., Schmid R. D., (1993), Biochim Biophys. Acta, 1168, 181-189.
19. Oi S., Sawada A., Satomura Y., (1967), Agric. Biol. Chem., 11, 1357-1366.
20. Alhir S., Markakis P., Chandan R. C., (1990), J. Agric. Food Chem., 38, 598-601.
21. Druet D., El Abbadi N., Comeau L. C., (1992), Appl. Microbiol. Biotechnol., 37, 745-749.
22. Yamaguchi S., Mase T., (1991), Appl. Microbiol. Biotechnol., 34, 720-725.
23. Okumura S., Iwai M., Tsujisaka Y., (1980), J. Biochem., 87, 205-211.
24. Pimentel M. C. B., Krieger N., Coelho L. C. C. B., Fontana J. O., Melo E. H. M., Ledingham W. M., Lima Filho J. L., (1994), Appl. Biochem. Biotechnol., 49, 59-74.
25. Iwai M., Okumura S., Tsujisaka Y., (1975), Agric. Biol. Chem., 39, 1063-1070.
26. Isobe K., Akiba T., Yamaguchi S., (1988), Agric. Biol. Chem., 52, 41-47.

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Summary

An extracellular lipase (glycerol ester hydrolases E.C. 3.1.1.3.) was isolated from a culture filtrate of *Penicillium citrinum*. The purification procedure included ammonium sulfate precipitation, ultrafiltration and chromatography on Octyl-Sepharose CL-4B. The enzyme was 400-fold purified with 9.66% yield. The molecular weight has been estimated by polyacrylamide gel electrophoresis under denaturing conditions at 26 000. On the other hand, lipase forms active dimers and tetramers aggregates as observed after native PAGE. Lipase from *Penicillium citrinum* showed a preference for triacylglycerols. It is non-specific and hydrolyzes each of the three ester bonds of triacylglycerols. The enzyme showed a maximum activity at pH 7.2 at 30°C and was stable in the range of pH 6.0-7.5 and the temperature of 10°C – 40°C.

Key words:
lipase, purification, *Penicillium*.

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