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Purification and Characterization of Lipase from *Trichosporon* sp. Y-11 and Its Use in Ester Synthesis of Unsaturated Fatty Acids and Alcohols

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Abstract A 28-kDa extracellular lipase (pI 8.7) was purified to homogeneity from the culture supernatant of *Trichosporon* sp. Y-11 by ammonium sulfate precipitation, DEAE-Sephadex A-50, Bio-Gel P-30, CM- Sephadex C-50, and Bio-Gel P-10 chromatographies. The purified enzyme exhibited a specific activity of 2,741 μmol/min/mg based on the hydrolysis of triolein, and the optimal hydrolysis activity was identified at pH 8.0 and 40°C. The enzyme activity was inhibited by Ag^+ and enhanced by Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, and Li^+. The enzyme activity exhibited for the hydrolysis of both tributyrin and trilinolein. The ester synthesis of unsaturated fatty acids with various alcohols catalyzed by the purified lipase in a non-aqueous medium or microaqueous system was also investigated. The esterification activity of the lipase increased with an increase of the carbon chain length in the alcohol. The synthesis rate of linoleic acid and oleyl alcohol was the highest with an optimal temperature and pH of 40°C and 8.0, respectively. The water content and agitation also affected the esterification activity of the lipase.

Key words: Lipase, *Trichosporon* sp., ester synthesis, unsaturated fatty acids, oleyl esters

Lipases (EC 3.1.1.3, glycerol ester hydrolase) catalyze the hydrolysis of triacylglycerols to glycerol and free fatty acids. Recently, a variety of new applications have merged, especially related to the enantioselective hydrolysis of esters [1, 9, 10, 21, 22], ester synthesis in organic solvents [3, 6, 11, 19], and the modification of sugars or chiral drugs [13, 16]. Many different kinds of microbial lipases have been reported [12], and received much attention due to their versatility and selectivity of catalysis and potential for use in the synthesis of novel compounds in industry [4, 8, 15, 20]. Because impure enzymes can cause many disadvantages in the field of biosynthesis, enzymes need to be purified and characterized before being used in biosynthesis.

Accordingly, this paper reports on the purification of a novel extracellular lipase from *Trichosporon* sp., using a multistep procedure, including ion-exchange chromatographies and gel filtration. The purified lipase was partially characterized and used for catalyzing the esterification of fatty acids and alcohols in a microaqueous system.

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Materials and Methods

Microorganisms and Enzyme Production

Trichosporon sp. Y-11 was isolated to produce lipase and maintained on YM slant [17]. The strain was cultured in a 2-l flask containing 1 l of a medium consisting of 2.0% soy bean powder, corn flour 2.0%, sucrose 1.0%, $(NH_4)_2SO_4$ 0.1%, K_2HPO_4 0.2%, and $MgSO_4 \cdot 7H_2O$ 0.01% (pH 6.0) at 28°C for 96 h on a reciprocal shaker. The culture broth was centrifuged for 15 min at 5,000 rpm and the supernatant obtained was used for enzyme purification.

Materials

The olive oil emulsion, DEAE-Sephadex A-50, Bio-Gel P-30, CM-Sephadex C-50, Bio-Gel P-10, linoleic acid, and linolenic acid were all purchased from Sigma Chemicals. The molecular weight markers for electrophoresis were obtained from Pharmacia (Uppsala, Sweden). All other chemicals were obtained from Tokyo Kasei Kogyo Co. Ltd.

Enzyme Assay

The lipase activity was determined titrimetrically, as described by Joeger and Haas [5]. The hydrolysis was performed in a 20 mM NaH_2PO_4-Na_2HPO_4 buffer, pH 8.0, at 40°C. The

Table 1. Purification of lipase from *Trichosporon* Y-11.

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Activity recovery (%)	Purification (fold)
Crude extract	4980.0	90000	18	100.0	1.0
Ammonium sulfate	547.2	82700	151	91.8	8.3
Biogel P-30	214.7	65040	302	72.2	16.7
DEAE Sephadex A-50	72.1	44630	619	49.6	34.2
CM-Sephadex C-50	8.0	19610	2451	21.8	136.1
Bio-gel P-10	5.2	14150	2721	15.7	150.3

reaction was started by the addition of the enzyme preparation. The acid released was continuously titrated at pH 7.0 by a 0.5 M NaOH solution with pH-Stat. One unit of lipase activity was defined as the amount of enzyme which liberated 1 mol of free fatty acid per min under the above conditions.

Protein Measurement

During column chromatography, the protein concentration in each fraction was monitored by measuring the absorbance at 280 nm. The protein concentration was also measured by the method of Hartree [2], using bovine serum albumin as the standard.

Enzyme Purification

The proteins in the *Trichosporon* sp. Y-11 culture filtrates were precipitated with ammonium sulfate (45% saturation) overnight at 4°C and recovered by centrifugation at 5000 ×g for 20 min. The precipitate was dialyzed against a 50 mM phosphate buffer (pH 8.0), then loaded onto a Bio-Gel P-30 column (2.6×100 cm) equilibrated and eluted with the same buffer. The enzyme fraction concentrated by ultrafiltration was loaded onto a DEAE-Sephadex A-50 column (2.6×30 cm) equilibrated with a Gly-NaOH buffer (pH 9.0). The column was eluted with a linear gradient of NaCl from 0 to 0.8 M in the buffer (total 500 ml). The active fractions eluting at around 0.2 M NaCl were pooled and concentrated by ultrafiltration, and then applied to CM-Sephadex C-50 equilibrated with a 50 mM sodium acetate buffer (pH 6.0). The elution was performed using the same buffer with a NaCl gradient at a flow rate of 30 ml/h. The active fraction was concentrated and applied to Bio-Gel P-10 (1.6×100 cm). The active fractions were pooled, lyophilized to a dry powder (2,000 U/g), and stored at −20°C. Purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing.

Ester Synthesis

The reaction mixture for the ester synthesis was composed of 5 mM unsaturated fatty acid (oleic acid, linoleic acid, or linolenic acid) and 0.1 g lipase in various alcohols, which were used as substrates and organic solvents. All reagents were placed in a 100-ml Erlenmeyer flask and incubated at 40°C for 2 or 6 h with constant agitation (250 rpm) by an oscillatory thermostat. The lipase was then removed from the mixtures by filtration, and a 50 ml acetone-ethanol mixture (1:1, v/v) was added to the filtrate to stop the reaction. The free fatty acid was titrated by 0.1 N NaOH. The esterification rate was calculated based on the reduction of fatty acids. The reaction products were extracted with diethyl ether and assayed by thin-layer chromatography (TLC). The TLC analysis was carried out on a silica gel F_{254} plate (Merck) in petroleum ether-diethyl ether-acetic acid (70:30:1, v/v), and then the components were visualized by spraying 50% sulfuric acid and heating at 105°C for 10 min.

RESULTS

Purification of Lipase

The purification results are summarized in Table 1. The enzyme was purified 150-fold with a recovery of 15.7%. The use of ammonium sulfate removed most proteins. The P-30 column chromatography removed several high molecular weight proteins and other molecules. DEAE-Sephadex and CM-Sephadex chromatographies separated certain proteins with similar molecular weights. However, there were still two proteins detected by UV detector which can not be differentiated from each other, but were successfully separated by high-resolution P-10 chromatography in the subsequent step.

Properties of Lipase

The specific activity of the purified enzyme was 2,721 U/mg protein, as determined by the spectrophotometric method. The purified lipase obtained by the procedure described above was a single band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with an M_r of 28 kDa (Fig. 1). The pI value was estimated to be 8.7 by isoelectric focusing.

Effects of Temperature on Activity and Stability

The optimum temperature for the activity of lipase was 40°C. The thermal stability was investigated by incubating the enzyme in a phosphate buffer at various temperatures for 2 h. The enzyme showed great stability up to 60°C.

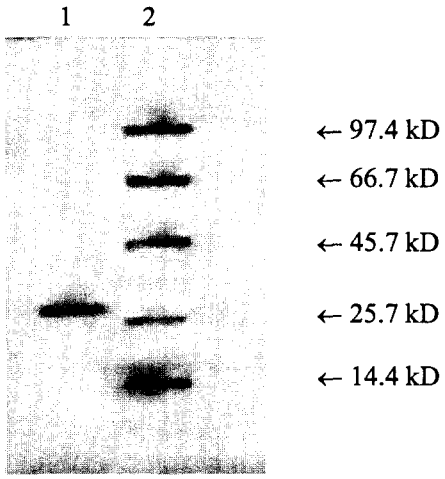

Fig. 1. SDS-PAGE graph of Trichosporon Y-11 lipase.
Lane 1: Y-11 lipase; lane 2: molecular weight marker.

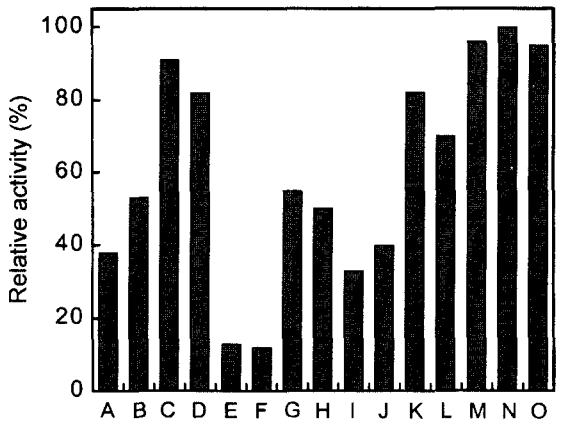

Fig. 3. Substrate specificity of Y-11 lipase.
A: acetin; B: propionate; C: butyrin; D: heptylin; E: 2-metyl butyrin; F: 2,2-dimetyl-divalerin; G: caprylate; H: dodecanate; I: Naphthyl acetin; J: benzoate; K: palmitin; L: stearin; M: olein; N: linolein; O: linolenin. The above 15 compounds were used as the substrates for substrate specificity studies.

Effects of pH on Activity and Stability

The enzyme was most active at pH range between 7.0 and 10.5, and the optimum pH was 8.0. The pH stability was studied by mixing the enzyme with different buffers from pH 5.0 to 11.0 for 24 h. The enzyme was very stable and retained 80% of its original activity in an alkaline pH up to 11.0 (Fig. 2).

Effect of Metals on Activity

The effect of various metal ions and surfactants on the enzyme activity was investigated by assaying the remaining activity after incubating the enzyme with 1 mM and 10 mM metal ions or surfactants (10 mM) for 1 h at 40°C. The activity was enhanced by Fe^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+}, Li^{+}, while Ag^{+} inhibited the enzyme activity. Among the surfactants tested, SDS showed an inhibitory effect, whereas non-ionic surfactants, such as Tween 80, Triton x-100, and Span 80, stimulated the enzyme activity significantly.

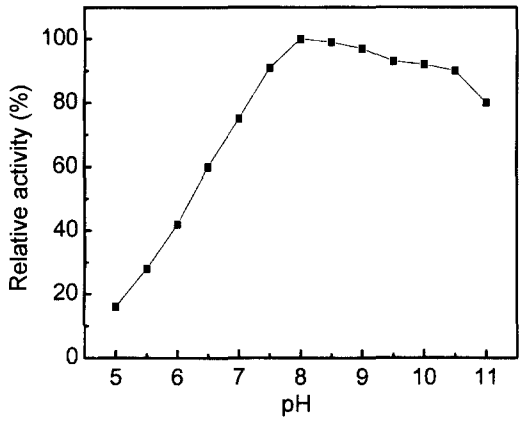

Fig. 2. pH stability of Y-11 lipase.
The pH stability was studied by mixing the enzyme with different buffers from pH 5.0 to 11.0 for 24 h.

Substrate Specificity of Lipase

As shown in Fig. 3, the lipase showed greatest activity with tributyrin, plus unsaturated long-chain fatty acid esters ($C_{16:1}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$) were also favored substrates.

Ester Synthesis from Oleic Acid, Linoleic Acid, or Linolenic Acid

The *Trichosporon* sp. Y-11 lipase catalyzed the synthesis of esters from various fatty acids and alcohols, however, the esterification of unsaturated long chain fatty acids, such as oleic, linoleic, or linolenic acids, was the main focus of the current study. Table 2 shows that *Trichosporon* sp. Y-11 lipase exhibited the highest esterification activity for the ester synthesis of unsaturated long chain fatty acids when compared with other lipases, and completed the reaction within a short time. Table 3 shows that the length of the carbon chain in the alcohols had a significant effect on the esterification rate. The esterification activity of the lipase increased with the chain length of the alcohols. It was also seen that the esterification rate increased in the order of primary and secondary alcohols. Tertiary alcohols were not esterified by the lipase. Figure 4 shows the effect of the carbon chain length and degree of unsaturation of the fatty acids on esterification. Medium and long chain fatty acids (C12-18) exhibited a high esterification potential with oleyl alcohol, especially for unsaturated fatty acids, as shown in Fig. 3. The esterification activity towards linoleic acid and oleyl alcohol was the highest.

Effect of Temperature and pH on Esterification Activity

The effects of temperature and pH on esterification rate were investigated with linoleic acid and oleyl alcohol as

Table 2. Comparison of esterification rates by different lipases.

Sources of lipases	Esterification rate$_{1\,h}$ (%)			Esterification rate$_{20\,h}$ (%)			Reference
	Oleate	Linoleate	Linolenate	Oleate	Linoleate	Linolenate	
Trichosporon Y-1	30.2	35.6	46.3	40.6	37.8	76.0	current study
Trichosporon Y-11	70.6	77.0	68.2	92	94.0	90.0	current study
Yeast S_9	36.3	37.8	35.5	47.5	40.2	39.7	17
Yeast S_{11}	40.2	42.1	45.8	44.3	47.8	47.9	17
Mould J_{8-2}	19.6	20.1	10.9	29.8	31.6	19.8	17
Mould M_2	60.5	45.5	62.5	75.3	71.2	78.3	17
Candida cylindracea	68.7	60.1	53.2	90.3	84.2	76.1	from Sigma
Porcine pancreatic	55.1	57.6	59.7	85.2	73.4	77.1	from Sigma

the substrates. The synthesis of oleyl linoleate proceeded in a very wide temperature range, from 0 to 100°C. The activity at 35°C was the highest, and 10% of this activity still remained at 100°C. The esterification activity of the lipase was significantly influenced by pH in organic solvents. The optimum pH for esterification was 8.0, which is the same as for a hydrolase.

Effect of Water Content

This was studied using oleic acid, linoleic acid, linolenic acid, and oleyl alcohol as substrates. As shown in Fig. 5, the water content greatly affected the esterification activity. The activity with a water content of 0.05% was the highest. With an increasing water content, the activity decreased. The activity dropped significantly when water content increased from 7% to 10%. The esterification rate increased to above 95%, when the water produced during the reaction was removed by the addition of a molecular sieve to the reaction system.

Effect of Agitation on Esterification

The effect of different agitating rates (100, 150, 200, 250 rpm) on the esterification of oleic acid, linoleic acid, or linolenic acid with oleyl alcohol, respectively, was investigated. The results showed that violent agitation was detrimental to the esterification activity of the lipase. An agitation rate of 200 rpm was the optimal for all three fatty acids. The esterification of linolenic acid and oleyl alcohol reached above 90% at this agitation rate, which was three times higher than that at 100 rpm (data not shown).

Table 3. Effects of various alcohols and unsaturated fatty acids on esterification rate.

Alcohol	Rate of esterification (%)		
	Oleic acid	Linoleic acid	Linolenic acid
Ethanol	15	17	13
Butanol	30	34	28
Octanol	45	40	35
Decanol	70	80	49
Dodecanol	85	88	65
Hexadecanol	80	90	82
Oleyl alcohol	92	94	90
Octadecanol	91	90	82
2-Butanol	23	25	18
2-Octanol	35	33	26
Cyclohexanol	45	56	48
Tertiary butanol	0	0	0
Terpineol	0	0	0

DISCUSSION

Although there have been many reports on the purification and characterization of lipases, including some from yeast, the lipases from *Trichosporon* sp. have not been studied as extensively as those from other yeasts. In the paper industry, an increasing importance is to use lipase in removing pitch from pulp, which must be carried out under

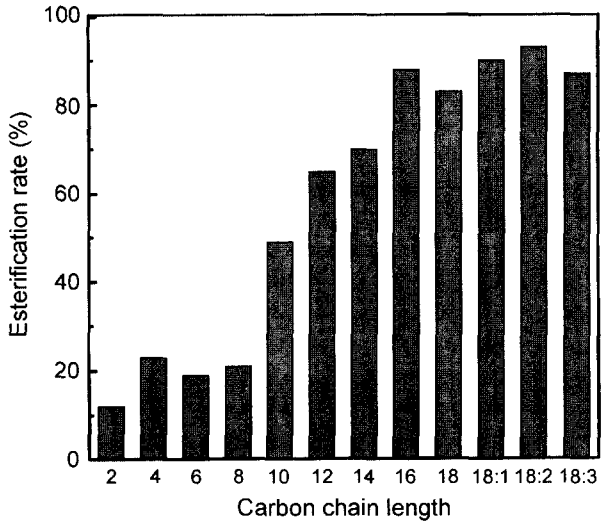

Fig. 4. Effect of carbon chain length and unsaturated degree of fatty acids on ester synthesis.

Short-chain (C2-8), medium-chain (C8-12), long-chain fatty acids (C12-18), and three unsaturated fatty acids (C18:1, C18:2, C18:3) were used to study the effect of carbon chain length and unsaturated degree of fatty acids in ester synthesis.

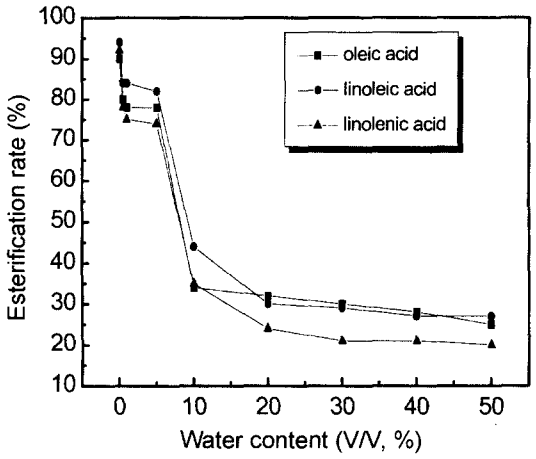

Fig. 5. Effect of water content on esterification activity.
Esterification rate was assayed every 0.025% in low water content range (0–2%) and every 5% in higher water content range (>2%).

alkaline conditions. In contrast to previous reports on most lipases from yeast [7, 14], the yeast lipase in the current study exhibited a high activity and stability in an alkaline pH (pH 8.0–11.0). The lipase also efficiently catalyzed the esterification of unsaturated fatty acids (oleic acid, linoleic acid, and linolenic acid) with various alcohols, including unsaturated oleyl alcohol. The esterification rates reached above 90% within a few hours, when all the reaction conditions (temperature, pH, water content, and agitation rate) were optimized. Oleyl oleate, oleyl linoleate, and oleyl linolenate are all wax esters, which can be used as high-quality lubricants, plasticizers, and cosmetics due to their good mobility and appropriate viscosity. The synthesis of various wax esters using an enzymatic method can overcome many disadvantages caused by chemical methods, such as the need for a high temperature and pressure, and more side reactions. Therefore, the synthesis of various wax esters by versatile microbial lipases in nonaqueous media or a microaqueous system is a new prospective technology. The current results show that the lipase from *Trichosporon* sp. Y-11 has great potential for industrial application.

One of the most important properties of lipases is their substrate specificity towards triacylglycerols. The substrate specificity of the current *Trichosporon* lipase was determined at 40°C for 30 min using the titrimetric method. The enzyme effectively hydrolyzed tributyrin and unsaturated long-chain fatty acid glycerides ($C_{16:1}$, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$). In the oleochemistry industry, enzymatic methods are used to hydrolyze oils and fats to produce unsaturated fatty acids instead of traditional high-temperature and high-pressure processes. Accordingly, the current lipase could be considered for this purpose due to its substrate specificity.

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