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Purification and characterization of a lipolytic enzyme active at low temperature from Norwegian *Typhula ishikariensis* group III strain

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Abstract

An extracellular lipolytic enzyme active at low temperature was purified from the culture filtrate of the snow mold fungus, *Typhula ishikariensis* group III, strain 6-1-1. The molecular mass of enzyme was approximately 83 kDa (SDS-PAGE). The lipolytic enzyme was most active for *p*-nitrophenyl palmitate at 30 °C (pH 9.0). The lipolytic activity was 23.4% of the maximum at 4 °C, the temperature of culture. Thus, the isolated *T. ishikariensis* lipolytic enzyme is thought to represent a new member of a group of enzymes active at low temperature.

Introduction

Typhula ishikariensis Imai is a psychrophilic fungal pathogen of winter cereals and grass in Norway (Årsvoll, 1975; Andersen, 1992), which has plant pathogenic activity under the snow cover. *T. ishikariensis* strains in Norway have been classified into three groups (group I, II and III) from genetic relationships by Matsumoto and Tronsmo (1995). Strains of group I and II grew normally at 10 °C. However, some strains of group III showed irregular growth at 10 °C. Culture morphology of group III resembled that of other taxa at 0 °C (Matsumoto et al., 1996; Hoshino et al. unpublished results). Therefore, strains of group III appeared to be better adapted to low temperature than those of other groups. This hypothesis was supported by the observation that group III strains predominated in Finnmark, a northern part of Norway (Matsumoto and Tronsmo, 1995).

Plant pathogenic fungi produce many kinds of macerating enzymes that cause the degradation of plant cuticle and cell wall during the infection. The cuticle is the external barrier for pathogen invasion, and the hydrolysis of cutin is an important step of plant infection. This reaction is catalyzed by cutinase and other lipolytic enzymes such as lipase and esterase (Kolattukudy, 1984) that are potential weapons of plant pathogens. *T. ishikariensis* penetrates into bentgrass leaves either through cuticles or stomata via single hyphae or an infection cushion formed on the host surface. When hyphae penetrate through an intact cuticle of epidermal cells, the host cell cuticle seem to dissolve enzymatically at penetration site (Ohshiman et al., 1995). Previously, other snow mold fungi *Typhula incarnata* and *Microdochium nivale* (syn. *Fusarium nivale*) have been shown to produce lipolytic enzymes (Mulanax and Huber, 1972; Hoshino et al., 1996). *T. ishikariensis* produces some cell wall digesting enzymes such as cellulase, hemicellulase and xylanase (Mulanax and Huber, 1970). However, it was not reported that the lipolytic enzyme production by *T. ishikariensis* had not been reported previously.

When snow mold infects host plants under the snow cover, extracellular lipolytic enzymes must be active at low temperature. Previously, we reported that the lipolytic enzyme of *Microdochium nivale* is a member of a group of enzymes active at low temperature (Hoshino et al., 1996) suggesting that Norwegian *T.*

ishikariensis group III strains may also produce low temperature-active extracellular lipolytic enzymes.

From the information available at present, the role of lipolytic enzymes in pathogenesis is still not clearly known and needs further investigation. As an important step towards a detailed analysis of the role of this activity in pathogenesis by molecular techniques, we purified and partially characterized an extracellular low temperature-active lipolytic enzyme from the strain of Norwegian *T. ishikariensis* group III.

Materials and methods

Fungal strains and media

Fifteen strains of *T. ishikariensis* group III were isolated in several areas in Norway (Matsumoto, Tronsmo and Shimanuki, 1996). Among them, strain 6-1-1 was selected to be used for this study on the basis of highest lipolytic activity. Strain 6-1-1 was collected by Sør-Varanger in Finnmark (northern Norway) in 1992. Cultures were maintained on potato-dextrose agar slants at 4 °C. The mesophilic fungal pathogen, *Fusarium oxysporum* f. sp. *lini* SUF 402 was obtained from the culture collection of Shinshu University. Lipolytic enzyme production medium was composed of 2% Tween 80, 1% $(NH_4)_2SO_4$, 0.1% yeast extract, 0.1% K_2HPO_4 and 0.1% MgSO4. The carbon sources added as a supplement to lipolytic enzyme production medium were various concentrations of glucose, soluble starch, sucrose, cellulose, xylan from oat spelt and wheat straw. Wheat straws were washed thoroughly with tap water to make them dust free, air dried and ground into powder before use.

Determination of lipolytic enzyme activity

Lipolytic activity was assayed by a colorimetric method (Winkler and Stuckmann, 1979) using *p*-nitrophenyl palmitate as a standard substrate. Ten ml of 2-propanol containing 30 mg of *p*-nitrophenyl palmitate was mixed with 90 ml of 25 mM Tris-HCl buffer (pH 9.0, *T. ishikariensis* or pH 7.0, *F. oxysporum*) containing 207 mg of sodium deoxycholate and 100 mg of gum arabic. Eight hundred μl of this freshly prepared substrate solution was warmed at 30 °C and mixed with 200 μl of enzyme solution. After 20 min, the reaction was stopped by adding 200 μl of 2 M sodium carbonate solution and released *p*-nitrophenol was measured by A_{410}. One unit of lipolytic enzyme activity was defined as the amount of enzyme releasing 1 μmole of free palmitic acid per minute at 30 °C.

Table 1. The effect of different carbon sources on the production of lipolytic enzyme by *T. ishikariensis*

Carbon source	Lipolytic activity (U/ml)	Day of maximum lipolytic activity
Glucose (2%)	0	–
Soluble starch (2%)	0	–
Sucrose (2%)	0	–
Cellulose (2%)	0	–
Xylan (2%)	0	–
Tween 80	0.45	30
Tween 80 + glucose (2%)	0	–
Tween 80 + glucose (1%)	0	–
Tween 80 + glucose (0.5%)	0.11	45
Tween 80 + glucose (0.25%)	0.39	40
Tween 80 + cellulose (2%)	0.41	28
Tween 80 + xylan (2%)	0.42	30
Wheat straw (1%)	0.97	45

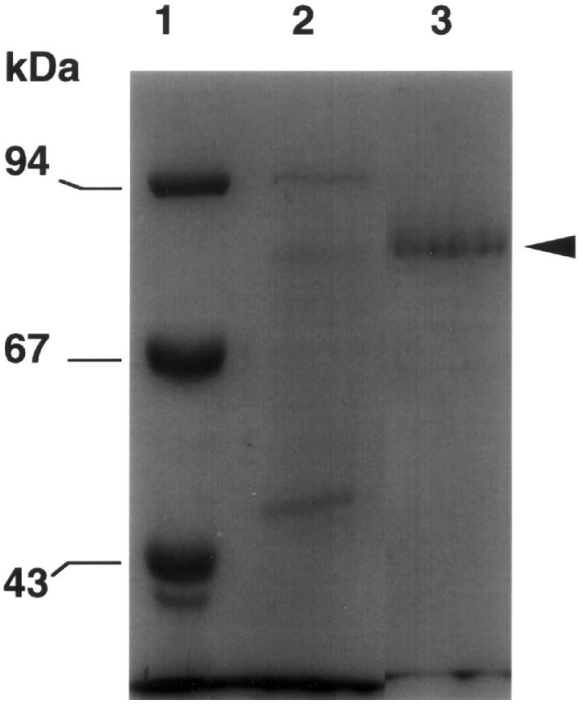

Figure 1. Molecular mass of *T. ishikariensis* lipase. Lane 1, molecular weight marker; 2, culture filtrate; 3, purified enzyme.

Table 2. Purification of lipolytic enzyme from *T. ishikariensis*

Purification step	Volume (ml)	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Recovery (%)
Culture filtrate	2000	2613	1176	0.45	100
$(NH_4)_2SO_4$ precipitate	18	13.4	375	28	32
Hydroxyapatite	5	3.2	282	88	24

Purification of lipolytic enzyme

One hundred ml *T. ishikariensis* cultures were prepared by inoculating 5 mm diam mycelial disc, cut from the margin of an actively growing colony in potato-dextrose agar plate, into the above lipolytic production medium followed by cultivation for 1 or 2 months at 4 °C with vigorous shaking. Mycelia were removed by filtration. When the culture filtrate was adjusted to 25% saturation with $(NH_4)_2SO_4$, a floating material containing lipolytic activity was formed. This was then dissolved into 50 mM Tris-HCl buffer (pH 8.5) containing 0.1 mM PMSF and dialyzed against the same buffer. The dialyzate was put on a column of hydroxyapatite (2.0 × 5.0 cm). The fraction containing lipolytic activity was stepwise eluted with the above buffer supplemented with 0.15 M NaCl. This preparation was the purified enzyme. Purification of *F. oxysporum* lipase was previously described (Hoshino et al., 1992). The protein concentration was determined using the BCA protein assay reagent (Pierce, IL, USA) and bovine serum albumin as standard. SDS-PAGE was used according to Laemmli (1970). The isoelectric point was measured by an isoelectro focusing gel electrophoresis (Bio-Lyte 3/10 Ampholyte, BIO-RAD, CA, USA).

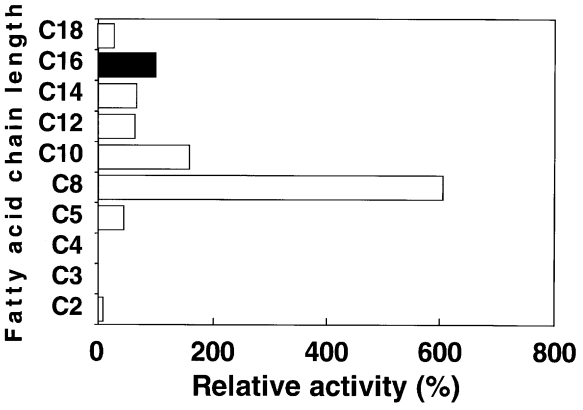

Figure 2. Fatty acid chain length specificity of lipolytic enzyme. Substrates were *p*-nitrophenyl fatty acid esters. Reaction mixtures contained 30 mg of each substrate, respectively. Relative activity of hydrolyses of each substrate (□) are basis of that of *p*-nitrophenyl palmitate (■).

Results and discussion

Lipolytic enzyme production by Typhula ishikariensis *group III strain*

Many fungal lipases are induced by lipid-related substances (Iwai, 1991). The same results were obtained in the case of *T. ishikariensis* 6-1-1 strain. The extracellular lipolytic activity was detected when Tween 80 was used as a sole carbon source, but not when sucrose and other sugars were used as carbon source (Table 1). Moreover, the appearance of lipolytic activity was delayed to 10–15 days from the date of inoculation when 0.5% glucose was added to the medium containing Tween 80. The increase in extracellular lipolytic activity was detected only during the late log phase of mycelial growth. Maximum lipolytic activity of culture filtrate was 0.45 U/mg·protein. Lipolytic enzyme production of *T. ishikariensis* was threefold higher than that of the pink snow mold, *Microdochium nivale* (Hoshino et al., 1996) and *T. incarnata* (Hoshino et al., unpublished results).

Molecular properties of lipase

When culture supernatant of *T. ishikariensis* 6-1-1 was adjusted to 25% saturation of $(NH_4)_2SO_4$, Tween 80 was separated from other medium components as a floating material. This material contained lipolytic activity probably due to an affinity of the lipolytic enzyme for Tween 80. All contaminating proteins were removed by the salt treatment. Lipolytic enzyme and Tween 80 were separated by hydroxyapatite column chromatography. Purified Norwegian *T. ishikariensis* lipolytic enzyme gave a single band on SDS-PAGE (Figure 1). Recovery of the enzymatic activity

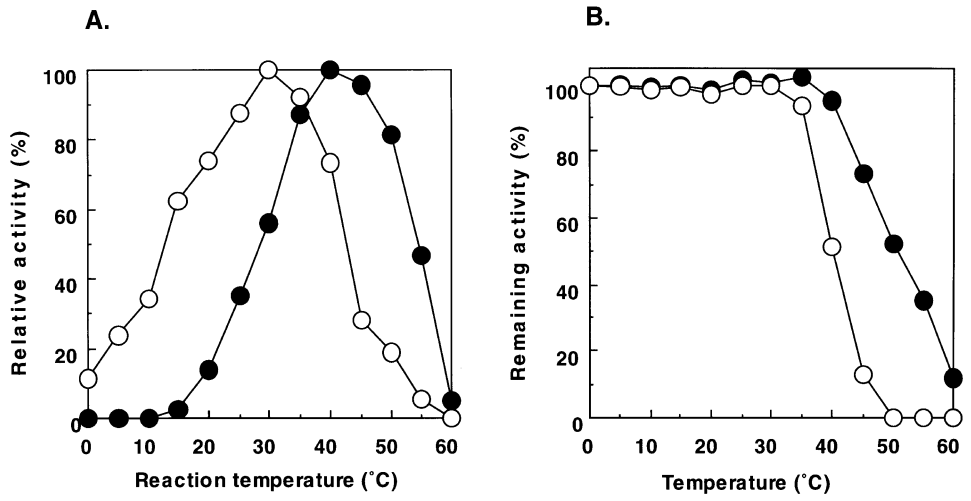

Figure 3. Effect of temperature on *T. ishikariensis* lipase. (A) Temperature dependence. Lipolytic activity was determined at optimum pH (25 mM Tris-HCl buffer) over 20 min. (B) Thermal inactivation. Purified enzyme solutions were incubated at various temperatures for 1 h and pH 7.5 (25 mM Tris-HCl buffer). The lipolytic activities of both enzymes were determined at optimal temperature and optimal pH, respectively. ○, *T. ishikariensis* lipolytic enzyme. ●, *F. oxysporum* lipase.

was 24%, and the specific activity of the purified lipolytic enzyme of Norwegian *T. ishikariensis* was 88 U/mg·protein (Table 2).

Molecular mass of the purified lipase was approximately 83 kDa as assessed by SDS-PAGE (Figure 1). The molecular mass of lipolytic enzymes was different to that reported here from other fungi and yeast. The pI value of the purified enzyme was calculated to be 8.2 (data not shown). The N-terminal amino acid sequence could not be determined probably because the N-terminal amino acid was blocked.

The optimum pH of the activity was pH 9.0. The lipolytic activity had significantly high stability in neutral and alkaline regions (pH 6.0 to 9.0 for 1 h). The purified enzyme was more active towards *p*-nitrophenyl esters with long and middle chain fatty acids (C8–C18) than with short chain fatty acids (C2–C5) (Figure 2). The properties of the *T. ishikariensis* lipolytic enzyme differ from those of the cutinases of mesophilic fungi. Mesophilic fungal cutinases catalyze hydrolysis of *p*-nitrophenyl esters of short and middle chain length fatty acids (C2–C8) but the esters of long chain acids gave lower maximal velocity values. Fungal cutinases of *F. solani* and *F. roseum* were reported to be inhibited by diisopropylfluorophosphate (Kolattukudy, 1984). However, ester bonds of long and middle chain fatty acids were hydrolyzed by the *T. ishikariensis* lipolytic enzyme, and the enzymatic activity was not inhibited by diisopropylfluorophosphate and PMSF (data not shown).

This enzyme showed unique characteristics as to the effect of temperature on activity. Many kinds of fungal lipase (Iwai, 1991) and cutinase (Kolattukudy, 1984) have optimal temperature range from 40 to 60 °C. Norwegian *T. ishikariensis* lipolytic enzyme had the highest activity at 30 °C. (Figure 3A). Furthermore, the enzymatic activity remained 11.4 and 23.4% of the maximum activity at 0 and 4 °C (cultivation temperature), respectively. Norwegian *T. ishikariensis* lipolytic enzyme had higher activity at low temperature than those of mesophilic fungi, but it had lower thermal stability than those of mesophile (Iwai, 1991; Hoshino et al., 1996). The lipolytic activity was reduced by about 50% at 40 °C when the enzyme solution was incubated for 30 minutes (Figure 3B). These results suggested that Norwegian *T. ishikariensis* lipolytic enzyme was a new member of the group of low temperature active enzymes. It is the first time that the isolation and characterization of a low temperature active lipolytic enzyme from psychrophilic microorganisms has been reported.

We are currently investigating the relationship between lipolytic activity and pathogenicity of *T. ishikariensis* as a problem yet to be solved in the future.

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