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Characterization of *Geotrichum candidum* lipase III with some preference for the inside ester bond of triglyceride

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Abstract. A new form of *Geotrichum candidum* lipase with a unique positional specificity was found to exist in the culture broth as a minor component together with the well-documented major form. Unlike the major form, which cleaves both the inside and outside ester bonds of triglyceride indiscriminately, the newly isolated form showed some preference for the inside (2-position) ester bond. The new enzyme was also characterized by its own fatty acid specificity, i.e., an outstandingly high activity towards triolein and methyl oleate among the simple triglycerides and fatty acid methyl esters tested. Moreover, the enzyme possessed a specific activity three times as high as the major form. Notable difference in circular dichroism spectra were observed between the two forms, indicating distinct conformational differences. Edman degradation revealed that the N-terminal sequence of the new form differed from that of the major form, thus demonstrating the existence of a novel lipase gene on the chromosome.

Introduction

Applied studies on lipase have become a subject of great interest ever since the enzyme proved to be able to remain catalytically active in nearly anhydrous reaction systems. Under these conditions the normal hydrolytic action of lipase is restricted because of the limited availability of water, and the enzyme can be used as an effective catalyst for a wide range of esterification (Tsujisaka et al. 1977; Okumura et al. 1979) and transesterification (Macrae 1983a; Kirchner et al. 1985). One potential advantage of using lipase-mediated reactions either in place of or in addition to chemical procedures resides in the specificity of the enzyme, i.e., fatty acid, positional (regio), and stereospecificities. These specificities are being exploited for the above reactions to elaborate valuable products that are difficult to obtain by chemical processes.

Positional specificity of lipase is of practical value in oleochemistry (Yokozeki et al. 1982) and organic synthesis (Therisod and Kilbanov 1986; Wang et al. 1988; Chopineau et al. 1988), and has long been studied. Lipases vary in positional specificity from rigorously 1,3-specific to completely non-specific via selective specificity (Machida 1984). Most of the mammalian and microbial lipases are considered to show overwhelming preference for the primary ester bonds (Sonnet 1988); they hydrolyse only outside ester bonds of triglyceride (1,3-specific). They can be used for the synthesis of primary esters (Okumura et al. 1979). In contrast, a few lipases are known to cleave all the ester bonds of triglyceride indiscriminately (non-specific). This type of enzyme can be used for the synthesis of both primary and secondary alcohol esters (Okumura et al. 1979). However, there are no authenticated reports on lipase releasing fatty acids from the inside position of the glycerol moiety alone (Macrae 1983b).

Geotrichum candidum lipase has been reported to be completely non-specific (Alford et al. 1964; Marks et al. 1968; Okumura et al. 1976; Tahoun 1987). We previously showed that the strain isolated in our laboratory (ATCC 34614) produced two forms of lipase (I and II), and that they were derived from different genes (Shimada et al. 1989, 1990; Sugihara et al. 1990). Subsequently, a larger scale hydrophobic interaction chromatography revealed that the microorganism secreted one major (lipase I), and three minor forms (lipase II, III, and IV) (Sugihara et al. 1991). Lipase IV was noteworthy in that it showed a unique positional specificity, i.e., some preference for the inside ester bond. Owing to its paucity, however, studies of the enzyme were restricted to the elucidation of fatty acid and positional specificities. Although lipase II and III were not completely resolved chromatographically, we have recently succeeded in separating the two forms, and have found that lipase III exhibits some preference for the inside ester bond, just like lipase IV. This paper deals with the isolation and characterization of lipase

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III, which is a novel form derived from a gene different from those of lipase I and II.

Materials and methods

Enzyme assay. Lipase activity was assayed at 30° C with olive oil (Wako Pure Chemical Industries, Osaka, Japan) as a substrate (Sugihara et al. 1990). Simple triglycerides and fatty acid methyl esters for the study of substrate specificity were obtained from Tokyo Chemical Industry, Tokyo, Japan.

Protein determination. Protein was determined in accordance with the method of Webster (1970) with bovine serum albumin as a standard.

Purification of lipase. The three initial purification steps from the culture supernatant of *G. candidum* (ammonium sulphate fractionation, DEAE-Sephadex A50 ion exchange chromatography, and Sephadex G100 gel filtration) were followed as described (Tsujisaka et al. 1973). Further purification was achieved by means of hydrophobic interaction chromatography. The ultrafiltration concentrate from Sephadex G100 gel filtration containing 598 mg of the protein was brought to 15% saturation with ammonium sulphate, and was put on a Butyl Toyopearl 650S column (2.7 × 21 cm; Tosoh, Tokyo, Japan) equilibrated with 10 mM acetate (pH 5.6) containing 15% saturated ammonium sulphate. The column was washed with three column volumes of the same buffer. Materials were then eluted with a decreasing gradient of 15–0% saturated ammonium sulphate in the same buffer at a flow rate of 20 ml/h. The second hydrophobic interaction chromatography was performed using a Bakerbond HI-propyl column (0.8 × 25 cm; J. T. Baker, Phillipsburg, USA) equilibrated with 10 mM acetate (pH 5.6) containing 10% ammonium sulphate. Elution was effected by decreasing the salt concentration from 10 to 0% at a flow rate of 1.5 ml/h.

Thin-layer chromatography. Positional specificity of lipase was examined by the method reported previously (Sugihara et al. 1991). Pure triolein was obtained by passing the commercial product through a Wakogel C200 silica gel column (2.5 × 50 cm, Wako Pure Chemical Industries, Osaka, Japan) with benzene as an eluent. Standard mono- and diolein were the products of Serdary Research Laboratories (London, UK).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). This was done according to Laemmli (1970) on a 7.5% acrylamide gel. Molecular mass standards were purchased from Bio-Rad (Richmond, Calif., USA). Protein bands were visualized by staining with Coomassie Brilliant Blue.

Amino acid analysis, Edman degradation, and carbohydrate determination. Prior to amino acid analysis and Edman degradation, the final enzyme preparation was further subjected to reversed phase HPLC on a Asahipak C4P-50 column (4.6 × 250 mm, Asahi Chemical Industry, Kawasaki, Japan). A gradient from 10 to 70% acetonitrile containing 0.05% trifluoroacetic acid was employed for elution. Amino acid analysis, removal of N-terminal amino acid, Edman degradation, and carbohydrate determination were carried out as described previously (Sugihara et al. 1990).

Circular dichroism (CD) measurements. Measurements of CD spectra were carried out at 25° C on a Jasco J500A spectropolarimeter (Japan Spectroscopic Co., Tokyo, Japan). Cells of 0.2 and 10 mm optical paths were used for the 200–250 and 250–310 nm regions, respectively.

Results

Purification of lipase

Hydrophobic interaction chromatography on a Butyl Toyopearl 650S column resolved the enzyme into one major (lipase I) and three minor (lipase II, III, and IV) forms (Sugihara et al. 1991). Since lipase II and III were not yet completely separated, each was subjected to the second hydrophobic interaction chromatography on a Bakerbond HI-propyl column. Figure 1 demonstrates that this procedure separated the two forms effectively. On SDS-PAGE each of the four forms gave a single band corresponding to molecular masses of 66 kDa (I), 66 kDa (II), 64 kDa (III), and 56 kDa (IV).

Table 1 summarizes the results of purification. Lipase I and II had nearly the same specific activities, whereas lipase III and IV possessed definitely higher specific activities than those of lipase I and II. In particular, lipase III showed activity three times as high as lipase I.

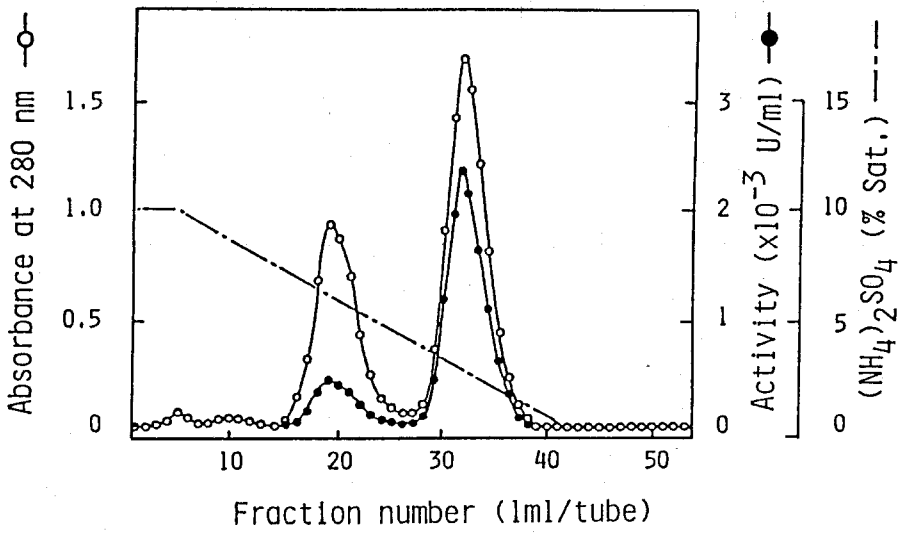

Fig. 1. Hydrophobic interaction chromatography of *Geotrichum candidum* lipase. The third fraction obtained by Butyl Toyopearl 650S column chromatography was rechromatographed on a Bakerbond HI-propyl column: U, units; Sat., saturation

Table 1. Purification of *Geotrichum candidum* lipase by means of two successive hydrophobic interaction chromatographies on a Butyl Toyopearl 650S and on a Bakerbond HI-propyl column

Step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
Applied	598	255800	428
Lipase I	453	199300	440
II	39	16900	433
III	14	18350	1310
IV	4	2880	720

Table 2. Amino acid composition and carbohydrate content of *G. candidum* lipase III, together with those of *G. candidum* lipase I and II deduced from their DNA sequences

Amino acid	No. of residues		
	Lipase III[a]	Lipase I	Lipase II
Asx	73.0	66	71
Thr	22.4	26	28
Ser	33.6	44	41
Glx	36.6	38	39
Pro	31.0	33	35
Gly	54.8	55	50
Ala	41.7	44	41
1/2 Cys[b]	4.0	5	4
Val	31.3	31	31
Met	10.6	12	12
Ile	22.2	25	25
Leu	48.2	49	51
Tyr	25.1	26	26
Phe	28.8	30	31
His	10.6	11	13
Lys	20.9	22	21
Arg	19.2	20	20
Trp[c]	7.2	7	5
Carbohydrate (%, w/w)	4.2	4.8	4.7

[a] Number of residues was calculated on the basis of a relative molecular mass of 57100

[b] Half-cystine was determined as cysteic acid after performic acid oxidation

[c] Tryptophan was determined after 4 M methanesulphonic acid hydrolysis

Amino acid composition, carbohydrate content, and N-terminal sequence

Table 2 shows the amino acid composition and carbohydrate content of lipase III, together with those of lipase I and II. The amino acid compositions of lipase I and II were deduced from the DNA sequences (Shimada et al. 1989, 1990). Both the amino acid composition and the carbohydrate content of lipase III were similar to those of lipase I and II. Edman degradation of the three forms was unsuccessful, suggesting the blocking of the N-terminus with pyroglutamic acid, as shown previously (Sugihara et al. 1990). Actually treatment of the proteins with pyroglutamate aminopeptidase provided access to Edman degradation.

Table 3. N-Terminal amino acid sequence of *G. candidum* lipase III, together with those of *G. candidum* lipase I and II

Enzyme	N-Terminal sequence
Lipase III	<Glu-Ala-Pro-Thr-Ala-Val-Leu-Asn-
Lipase I	<Glu-Ala-Pro-Arg-Pro-Ser-Leu-Asn-
Lipase II	<Glu-Ala-Phe-Pro-Pro-Ala-Val-Leu-

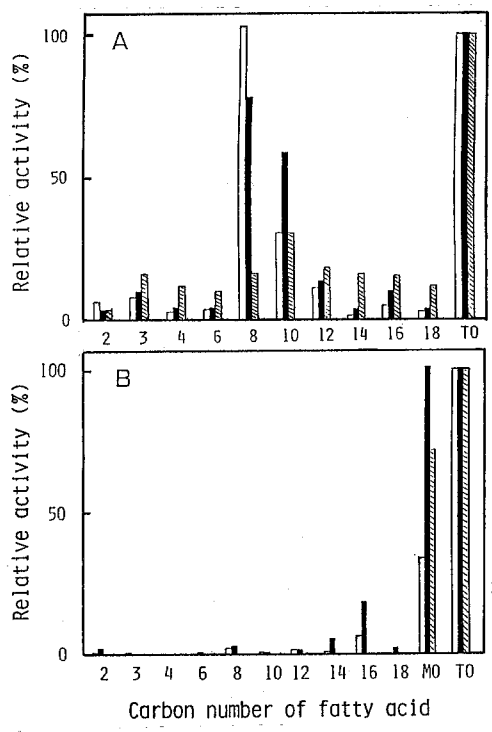

Fig. 2A, B. Enzyme activities of *G. candidum* lipases towards various simple triglycerides (**A**) and fatty acid methyl esters (**B**). The activity on each substrate was measured at 30° C in 50 mM phosphate buffer (pH 7.0) for 30 min, and is expressed as a percentage of that on triolein: □, lipase I; ■, lipase II; ▨, lipase III; TO, triolein; MO, methyl oleate

Table 3 represents the N-terminal amino acid sequences of the three enzymes. Lipase III proved to have an N-terminal sequence distinct from those of lipase I and II. The N-terminal sequences of lipase I and II agreed with those already reported.

Fatty acid specificity

Figure 2 shows the relative activities of each form towards various simple triglycerides and fatty acid methyl esters. Lipase I exhibited outstanding activities on tricaprylin and triolein. Lipase II acted well not only on tricaprylin and triolein, but on tricaprin and methyl oleate. On the other hand, lipase III showed much lower activity on tricaprylin, but high activity on triolein and methyl oleate.

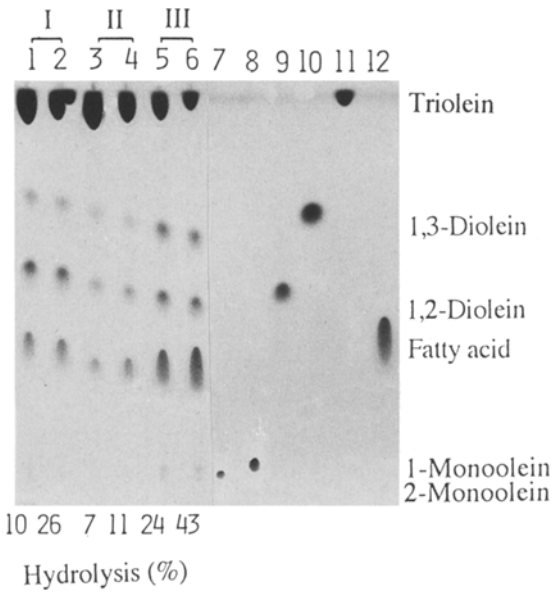

Fig. 3. Thin-layer chromatography of the partial hydrolysis products obtained by *G. candidum* lipases acting on triolein: *lanes 1* and *2*, with lipase I; *lanes 3* and *4*, with lipase II; *lanes 5* and *6*, lipase III; *lane 7*, 2-monoolein; *lane 8*, 1(3)-monoolein; *lane 9*, 1,2(2,3)-diolein; *lane 10*, 1,3-diolein; *lane 11*, triolein; *lane 12*, oleic acid

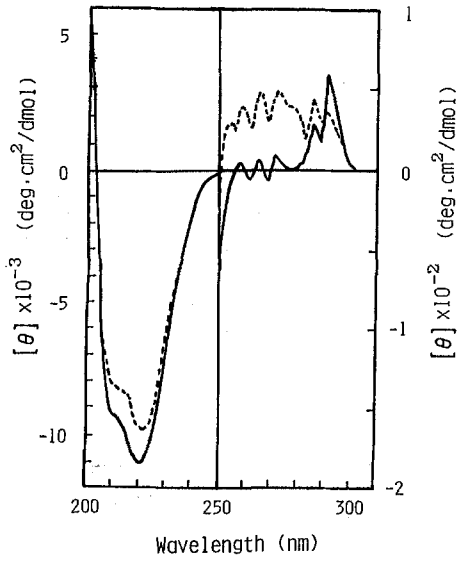

Fig. 4. Circular dichroism spectra of *G. candidum* lipase. The spectra were measured in 10 mM phosphate (pH 7.0) at 25° C: ——, lipase I, II; – – – –, lipase III

Positional specificity

Figure 3 shows thin-layer chromatograms of the partial hydrolysis products obtained by lipase I, II, and III acting on pure triolein. The extents of hydrolysis ranged from 10 to 43%. The spontaneous acyl group migration was thought negligible because of the short hydrolysis time. When lipase I was used, the amount of 1,2(2,3)-diolein formed at 26% hydrolysis was 2.2-fold that of 1,3-diolein, suggesting that the enzyme cleaved all the ester bonds at nearly the same rates, as claimed by several authors (Alford et al. 1964; Marks et al. 1968; Okumura et al. 1976; Tahoun 1987). Similar results were obtained with lipase II. In contrast, lipase III produced 1,3- and 1,2(2,3)-dioleins in about equal amounts (1:1.1) at 24% hydrolysis. At the same hydrolysis level, the amount of 1(3)-monoolein obtained with the aid of lipase III was 3.6-fold that of 2-monoolein. These results indicate that lipase III hydrolyses the 2-position ester bond nearly twice as fast as the 1(3)-position ester bond.

CD spectra

The CD spectra of lipase III greatly differed from those of lipase I and II over the peptide and aromatic regions (Fig. 4). The spectra of lipase I and II were practically indistinguishable. The trough at 222 nm of lipase III was obviously less deep as compared with that of lipase I, indicating that lipase III contains less α-helix than lipase I.

Other enzymatic properties

Lipase III showed its optimum pH at around 6.0, and optimum temperature at 40° C. The enzyme was stable between pH 3.5 and 9.0 for 19 h at 25° C. The enzyme retained all its activity up to 40° C for 30 min at pH 7.0.

Discussion

Two successive hydrophobic interaction chromatographies after ion exchange chromatography and gel filtration have demonstrated that *G. candidum* produces four chromatographically different forms of lipase. The first two fractions (lipase I and II) corresponded to the isozymes already characterized (Sugihara et al. 1990). The third fraction (lipase III) showed pH and temperature optima similar to those of lipase I and II, but possessed a specific activity three times as high as these enzymes. With regard to fatty acid specificity, we revealed in this study that lipase III exerted its activity exclusively on triolein and methyl oleate. The specificity was different from those of lipase I,II and IV (Sugihara et al. 1990, 1991). Although amino acid analysis failed to discriminate lipase III from lipase I and II, Edman degradation demonstrated that lipase III had an N-terminal sequence that is different from those of lipase I and II. From these data, we concluded that lipase III was derived from a gene distinct from those of lipase I and II. At this stage, however, it still remains unknown if lipase III and IV are derived from different genes; they show different fatty acid specificities but very similar positional specificities.

As mentioned earlier, positional specificity of lipases from different living organisms has been the subject of academic and applied research. Lipase from var-

ious *G. candidum* strains is one of the enzymes widely investigated with a view to isolating multiple forms. These forms are differentiated from each other on the basis of molecular mass, fatty acid specificity, carbohydrate content, or isoelectric point. The fatty acid specificity of lipase III of this study is similar to those of lipase B from *G. candidum* CMICC 335426 (Sidebottom et al. 1991), of lipase from *G. candidum* NRRL Y-553 (Baillargeon and McCarthy 1991), and of 57-kDa lipase from *G. candidum* ATCC 66592 (Jacobson and Poulsen 1992). However, no attention has been directed towards positional specificities of these forms since the reports by Alford et al. (1964), Marks et al. (1968), Okumura et al. (1976), and Tahoun (1987). Veeraragavan et al. (1990) have isolated two distinct lipases from the same strain as we used in this study, but have not mentioned their positional specificities. Thus, the newly characterized lipase III as well as lipase IV should be attractive enzymes with the unique positional specificity between 2-specific and non-specific.

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