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Original Article

Amino acid analysis of lipases from oil pollutant isolates: *Cunninghamella verticillata* and *Geotrichum candidum*

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Abstract

Lipase is an enzyme commonly used in food, dairy, and other industries. Fungal lipases are more commonly used due to their secretion and easier production. Analyses of the amino acid composition of microbial lipases will hasten their potential usage in industrial applications. In this study, the major amino acid compositions of lipases secreted by oil pollutant isolates (*Cunninghamella verticillata* and *Geotrichum candidum*) enriched with fatty substances were analyzed by high performance liquid chromatography. Among eight major amino acids found in these lipases, histidine and ornithine were predominant. Lysine was absent from lipase generated by *C. verticillata*, while glutamine was absent from that produced by *G. candidum*. Conversely, glutamic acid, asparagine, histidine and arginine were present in slightly higher amounts in *G. candidum*. However, a slight decrease in aspartic acid and ornithine was observed in *G. candidum*. Analyses of the amino acids composition of lipase can potentially facilitate to predict the nature of this enzyme.

Keywords: amino acid, *Cunninghamella verticillata*, *Geotrichum candidum*, high performance liquid chromatography, lipase

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1. Introduction

Lipases are defined as triacylglycerol acylhydrolases, which are industrially important water-soluble enzymes that catalyze the hydrolysis of long-chain triglycerides. Lipases catalyze the hydrolysis of ester bonds of triglycerides, diglycerides and monoglycerides into fatty acids and glycerol. Additionally, these enzymes play important roles in the production of biopolymers, biodiesel, pharmaceuticals, agrochemicals, cosmetics, flavors, and in the textile, detergent, food, pulp, fat, and oleochemical industry due to their substrate specificity and stability (Singh & Mukhopadhyay, 2012). Fungal lipases can be easily obtained and their low cost makes them preferable over bacterial lipases. Fatty substances such as oil and dairy products harbor a variety of fungal species that have the potential to secrete lipases capable of degrading these substances. Predicting lipase sequences from fungal species will hasten lipase production in the industries. Various extracellular productions of lipases have been well documented in a number of fungi, including zygomycetes (Akhtar *et al*., 1983), hyphomycetes (Chandar *et al*., 1980), yeasts (Tsujisaka *et al*., 1977), ascomycetes (Oso, 1978), and coelomycetes (Reddy & Reddy, 1983). Several fungal species have been found to have lipolytic activity, including *Mucor* spp. (Jensen *et al*., 1983), *Lipomyces starkeyi* (Sztajer *et al*., 1988), *Rhizopus* spp. (Sztajer *et al*., 1988; Thota *et al*., 2012), *Geotrichum candidum* (Gopinath *et al*., 2003), *Penicillium* spp. (Salihu *et al*., 2011), *Acremonium strictum* (Okeke & Okolo, 1990), *Candida rugosa* (Wu *et al*., 1990), *Humicola lanuginosa* (Lizumi *et al*., 1990), *Cunninghamella verticillata* (Gopinath *et al*., 2002), and *Aspergillus* spp. (Gopinath *et al*., 2000).

Amino acids are the major constituents of enzymes, being their second largest component after water. Analyses of the amino acid composition of the lipases, especially microbial secretions, will improve production of industrially important lipases. Amino acids are biologically important organic compounds containing -NH_2 and -COOH groups, as well as other vital elements such as carbon, hydrogen, oxygen, and nitrogen. Structurally, amino acids are classified as alpha- (α), beta- (β**)**, gamma (γ) or delta- (δ) amino acids. Categories include aliphatic, acyclic, aromatic, hydroxyl or sulfur containing amino acids. Lipases have been studied for over 150 years (Bernard, 1849); however, the entire primary amino acid lipase sequence was not revealed until 1981 (De Varo *et al*., 1981). The sequence of proteolytic peptides derived from lipoprotein lipase formed the basis of our preliminary understanding of these compounds in 1986 (Ben-Avram *et al*., 1986). Amino acid sequencing of enzymes permitted cDNA cloning, gene expression and crystallization (Wong & Schotz, 2002).In addition to direct sequence comparisons, amino acid sequencing has also expanded the protein structure database for the recognition of a superfamily of enzymes (Ollis *et al*., 1992).

The basic building block of amino acids that form the three-dimensional structure of a native enzyme is an essential starting point for investigations of structure-function relationships. Fungal lipases from different species have been solved by X-ray crystallography (Derewenda *et al*., 1994), and resolution of protein structure has generated excitement and stimulated further research. Detailed knowledge regarding the amino acid composition of lipase along with the structures enables new design of enzymes well-suited for future industrial applications. Therefore, in this study, potential fungal isolates from an environment enriched with fatty substances were subjected to lipase purification as described before (Gopinath *et al*., 2002, 2003). The amino acid composition of these lipases was further analyzed by high-performance liquid chromatography.

2. Materials and Methods

Isolation of lipolytic fungal species, including the production and purification of lipases, was conducted as described in on our previous studies (Gopinath *et al*., 2002, 2003, 2005). In brief, the extracellular lipases from *C. verticillata* and *G. candidum* were produced in yeast-extract medium supplemented with carbon and nitrogen sources. The secreted lipase in the presence of olive oil was precipitated by ammonium sulphate and further purified by ion-exchange (DEAE-Sephadex) and gel-filtration columns (Sephadex G-100 and G-200). The purity of lipase fractions were analyzed by native- and SDS-PAGE.

2.1 Gel filtration

Sephadex G-100 and G-200 gel columns (74 x 1.5 cm) were used to determine the molecular weight of the enzyme. The enzyme fractions collected upon DEAE-Sephadex column chromatography (Gopinath *et al*., 2002) were concentrated and loaded onto a Sephadex G-100 column that had been conditioned with 0.01 M acetate buffer (pH 5.0). Elution was carried out in the same buffer at a flow rate of 15 mL/h, after which the active fractions were collected. The elution volume (V_e) for each protein was determined by measuring the absorbance at 280 nm. The void volume (V_o) was determined using Blue Dextran 2000. Standard proteins used were phosphorylase B (MW 97.4 kDa), Bovine Serum Albumin (66 kDa), Ovalbumin (45 kDa), Chymotripsin (25 kDa) and Ribonuclease (13.7 kDa). The straight-line plot obtained was used for determination of the molecular weight of purified lipase.

2.2 Ultraviolet absorption spectrum of lipases

UV absorption spectra of the purified lipases were analyzed at varying wavelengths (220-340 nm) using a Philips Spectrophotometer (model PU 8700).

2.3 Assay for lipase

The purified extracellular lipase was assayed quantitatively using p-nitrophenylpalmitate as the substrate (Gopinath *et al*.,2002, 2003). Briefly, 10 mL of isopropanol containing 30 mg of 4-nitrophenyl palmitate was mixed with 90 mL of 0.05 M phosphate buffer (pH 8.0) containing 207 mg of sodium deoxycholate and 100 mg of gum arabic. Next, 2.4 mL of this freshly prepared substrate solution was prewarmed at 37 °C and then mixed with 0.1 ml of cell free supernatant fluid. After 15 min. of incubation at 37 °C, the optical density (O.D.) at 410 nm was measured against an enzyme free control. One enzyme unit was defined as 1 µmol of 4-nitrophenol

enzymatically released from the substrate $ml^{-100}min^{-1}$. The lipase of *Mucor miehei* was used as the standard for comparison and obtained from Sigma-Aldrich.

2.4 Estimation of amino acids composition of purified lipase by high performance liquid chromatography

Amino acid composition of the purified lipase was analyzed by high performance liquid chromatography (HPLC) using the Ortho-Phthalaldehyde (OPA) method. To prepare OPA, 50 mg of anhydrous OPA was dissolved in 2 mL methanol, after which 8 ml of borate buffer and 50 µL of 2-mercaptoethanol were added. The reagents were prepared fresh before each use and kept in the dark. The purified lipase was acid hydrolyzed before being loaded onto the column (Natarajan, 1995). Next, 50 mg of dry powdered sample was weighed accurately into a labeled clean glass ampule (10 mL capacity), after which 5 ml of 6N HCl was washed down the sides of the ampule and the ampule was evacuated and sealed. The sample was subsequently hydrolyzed with 6 N HCl at 110 °C for 22 hrs and cooled, centrifuged at 2,000 rpm for a few minutes, then filtered through acid washed filter paper. The filter paper was rinsed several times with 1 % HCl, after which the filtrate was pooled and diluted to 25 mL with 1% HCl. Next, 5 mL aliquots of the filtrate were evaporated in a flash evaporator, after which traces of HCl were removed by placing the samples over NaOH pellets in a vacuum desiccator. The contents were then dissolved and the volume was increased to 5 mL with diluent. Next, the samples were passed through a 0.22-micron membrane filter and subjected to amino acid analysis by HPLC. The operating conditions of HPLC were as follows: column (C18, 4.6 x 250 mm, 5 µm packing); mobile phase A (0.1 M acetate buffer); mobile phase B (3% Tetrahydrofuran in methanol); flow rate (1.5 mL/min); gradient (10-42% B for 15 min/42% B for 10 min/42-50% B for 3 min/50-70% B for 7 min/70-90% B for 4 min/90-100% B for 1 min/100% B for 2 min/100-10% B for 1 min/10% B for 2 min); detector (fluorescence, 9 µL flow cell FL-2); excitation filter (305-395 nm); emission filter (430-470 nm); sensitivity (0.005 Abs). A standard amino acid kit was used to identify individual amino acids in the sample both qualitatively and quantitatively. Amount of amino acids present in each sample was expressed in µg/g sample.

2.5 Determination of cysteine (S-S)

The cysteine content of the purified amino acid was measured using the method described by Ramakrishna *et al*. (1979). Briefly, 4 mL of test solution was amended with 2 mL of 0.2% $MgCl_2$ solution and 3 mL of 0.01 M potassium dichromate solution successively. The sample was then diluted to 25 mL with distilled water in a calibrated volumetric flask, after which the absorbance was measured at 510 nm following 160 min of incubation against a corresponding reagent blank prepared in the same manner.

3. Results

Two fungal species, *C. verticillata* and *G. candidum*, recovered from the oil-mill effluent containing fatty substrates were selected for this study. The lipase from these organisms was extracted and purified (Figure 1). The native- and SDS-PAGE profiles of the purified lipases from *C. verticillata* and *G. candidum* are displayed in the supplementary Figure 1 and 2. The purified lipase was then confirmed by spectrophotometric analyses using 4-nitrophenyl palmitate as the substrate, after which its amino acid composition was determined.

Figure 1. Collection site polluted with fatty substances from oil-mill effluent.

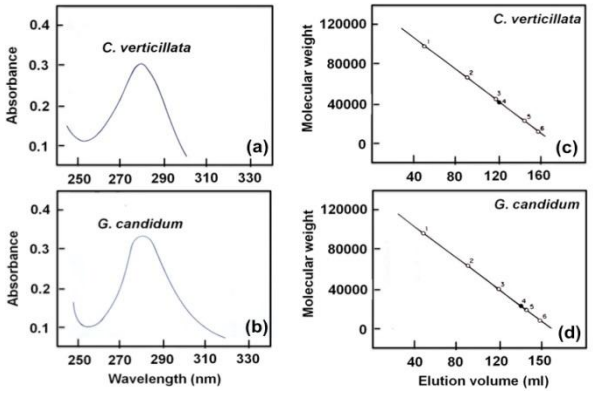


Figure 2. Ultraviolet absorption spectrum of purified lipases based on scanning performed at wavelengths ranging from 220-340 nm (a) *C. verticillata* (b) *G. candidum*. Determination of molecular weight of purified lipase from (c) *C. verticillata* (d) *G. candidum.* Sephadex G-100 was used to purify the lipase from *C. verticillata* at final step, whereas in the case of *G. candidum* Sephadex G-200 was used. Elusion volumes for these lipases were 120 and 135 ml, respectively.

3.1 Ultraviolet absorption spectrum of lipases

The ultraviolet (UV) absorption spectra of the purified lipase (1 mg/mL) from both fungal species were measured in phosphate buffer, pH 7.5, at different wavelengths. The results showed that the UV absorption spectra of lipase from both species are of typical protein spectra, with the maximum absorbance occurring at 280 nm and the minimum at 260 nm (Figure 2a and b). Before further analyses, the lipolytic activity was determined as ~10 and 45 U/mg for *C. verticillata* and *G. candidum*, respectively. The obtained lipase

activities were compared with the standard lipase from the commercial source. The lipase standard from *Mucor miehei* has displayed the enzymatic activity to be ~40 U/mg in 1/100 diluted form.

3.2 Molecular weight determination of lipase

The molecular weights of the lipase from both species were determined by gel filtration chromatography, which suggested molecular weights of 42±2 and 28±2 kDa, respectively (Figure 2c and 2d; Table 1). Gel scan diagrams of the reference proteins and purified lipase from *C. verticillata* and *G. candidum* are shown as Figure 3a and b. These studies yielded information regarding the length of the amino acid sequence of the purified lipases. The complete amino acid sequence of *C. verticillata* is not available; however, we displayed the available information from the online databank for the lipase from *Geotrichum candidum* (Figure 4a and 4b). As stated elsewhere, lipases are varying among the stains based on their adapted habitat. These variations make the differences in the molecular weight of lipases from different strains. The purified lipases from *C. verticillata* and *G. candidum* were analyzed for the abundance of the major amino acids composition.

Table 1. Determination of molecular weight

Kn (Known)/Unkn (Unknown) protein	Molecular weight	Log MW	Rf
Kn-Blue Dextran	200,000	5.16	0.082
Kn-Phospharylase B	97,400	4.98	0.259
Kn-Bovine Serum Albumin	66,000	4.82	0.38
Unkn-lipase from *C. verticillata*	49,100	4.67	0.49
Kn-Ovalbumin	45,000	4.65	0.52
Unkn-lipase from *G. candidum*	31,996	4.47	0.646
Kn-Chymotripsin	25,000	4.3	0.759
Kn-Ribonuclease A	13,700	4.2	0.84

Rf values were calculated by plotting between Kav and log molecular weight.

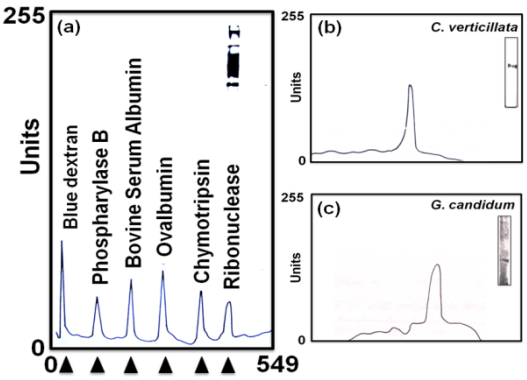
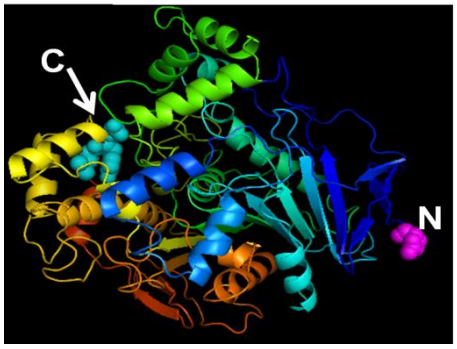


Figure 3. Scanning laser densitometry analysis (a) protein markers (b) lipase of *C. verticillata* (c) lipase of *G. candidum.* Sephadex G-100 was used to purify the lipase from *C. verticillata* at final step, whereas in the case of *G. candidum* Sephadex G-200 was used.

4a).



4b).

Figure 4. Analyses of lipase from *G. candidum.*(a) Amino acid sequence of *G. candidum* (GenBank accession No. AFO66622). (b) Three dimensional crystal structure of *G. candidum* (PDB code-1THG).

3.3 Amino acid analysis of purified lipases by HPLC

The purified lipase samples were hydrolyzed and amino acids were determined using HPLC. The numbers of amino acid residues per µg/g of the purified lipases analyzed are presented in Table 2 and Figure 5. It should be noted that cysteine and proline are not included because the HPLC system used for this study could not detect these amino acids. Therefore, the presence of cysteine was measured by another method. Eight major amino acids were found to be present in the purified lipase from *C. verticillata* and *G. candidum*. Among the major amino acids, histidine and ornithine were present in abundance, and ornithine was predominant in both of these lipases. Lysine (basic amino acid) was absent from the lipase produced by *C. verticillata*, while glutamine was absent from that produced by *G. candidum*. Nevertheless, the lipase from *G. candidum* showed the presence of lysine. Conversely, glutamic acid, asparagine, histidine and arginine were present in slightly higher amounts in *G. candidum* than the lipase from *C. verticillata*. However, a slight decrease in aspartic acid and ornithine was noticed in *G. candidum*. The

Table 2. Amino acid composition of the lipases from *Cunninghamella verticillata* and *Geotrichum candidum*

Amino acid		*Cunninghamella verticillata*		*Geotrichum candidum*	
	Type	Amount (µg/g)	%	Amount (µg/g)	%
Aspartic acid	Acidic	2.87±0.3	8.84	2.3±0.3	6.73
Glutamic acid	Acidic	1.86±0.1	5.72	2.48±0.3	7.26
Asparagine	Acidic	0.56±0.01	1.7	0.7±0.2	2.05
Histidine	Basic	8.01±0.5*	24.71	9.18±0.6*	26.86
Arginine	Basic	1.93±0.1	5.94	2.44±0.3	7.13
Ornithine	-	15.33±1.0*	47.26	14.38±0.9*	42.06
Lysine	Basic	0	0	2.71±0.3	7.92
Glutamine	-	1.89±0.1	5.83	0	0
Cysteine	-	2.6±0.3	NC	2.3±0.2	NC

Cysteine was calculated by the method described by Ramakrishna *et al*. (1979). NC-not calculated; * indicates the significant amount

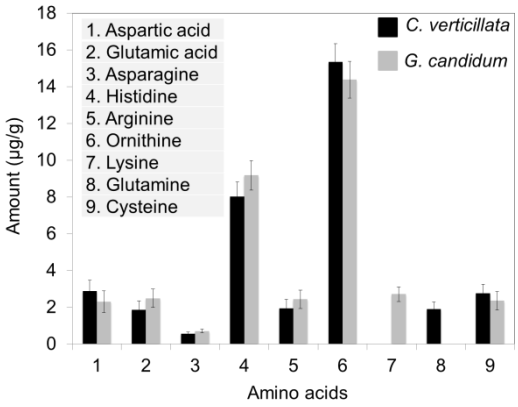


Figure 5. Graphical representation of amino acid comparison of lipases from *C. verticillata* and *G. candidum.*

lipase from both species was also found to have a higher molar level of basic amino acids in *C. verticillata* (77.91%) and in *G. candidum* (83.97%) than acidic amino acids in *C. verticillata* (22.09%) and *G. candidum* (16.03%) (Table 2, Figure 5). The sulfur-containing amino acid, cysteine, was found to be 2.76 µg/g and 2.34 µg/g in *C. verticillata* and *G. candidum*, respectively. The other sulfur containing amino acid, methionine, was not present in the hydrolyzate.

4. Discussion

Two fungal species (*C. verticillata* and *G. candidum*) found to produce lipases based on the Rhodamine-B method and by cultivation on 4-nitrophenyl palmitate were isolated from oil-mill effluent (Gopinath *et al*., 2002, 2003, 2005; Lee & Rhee, 1993). The secretion of lipase from these fungi was conducted under optimal conditions and the extracted lipase was purified (Gopinath *et al*., 2002, 2003). UV absorption spectra of lipases from *C. verticillata* and *G. candidum* showed the maximum absorbance spectrum at 280 nm. Additionally, the molecular weights determined by the gel filtration method (42±2 and 28±2 kDa, respectively) were comparable to those determined by SDS-PAGE (49±2 and 32±2 kDa, respectively) (Gopinath *et al*., 2002, 2003).

Purification of enzymes is known to be a successful strategy for determination of the composition of amino acids in a given protein sample and for structural studies (Saxena *et al*., 2003). In this study, the abundance of the major amino acids composition of lipases from *C. verticillata* and *G. candidum* was analyzed. Amino acids are monomer units of proteins, and the types of amino acids present, the linkage order, and the spatial relationship of one amino acid to another determine the biological properties of the proteins. Therefore, to comprehend the protein chemistry, information regarding the amino acids composition of lipases from both species is essential. Eight major amino acids were found to be present in the purified lipase from *C. verticillata* and *G. candidum*,with ornithine being most abundant, followed by histidine. The ratio of polar to apolar amino acid residues was zero since the lipase of *C. verticillata* and *G. candidum* belonged to a group of proteins with polar residues. Contrary to the present findings, Sztajer *et al*. (1992) reported that lipase from *Penicillium simplicissimum* belonged to a group of proteins with apolar residues (aspartic acid, serine, glycine, valine, isoleucine, threonine, histidine, arginine and cysteine). Sidebottom *et al*. (1991) found that the amino acid level of the lipase I and lipase II from *G. candidum* were 84% identical, and these enzymes showed some similarity (45%) to an extracellular lipase from *Candida rugosa* (Shimada *et al*., 1989).

Both lipases were reported to be inhibited by the cysteine specific inhibitor p-CMB, and the lipase from these two species is characterized as an enzyme with sulfur containing amino acid (Gopinath *et al*., 2002, 2003). A small amount of SH-containing amino acid was observed during amino acid analysis, which explains the inhibition of lipase activity by the cysteine specific inhibitor, p-CMB, reported for the lipase from *P. simplicissimum* by Sztajer *et al*. (1992). The consensus motif of G-X1-S-X2-G is present in most lipases, and the catalytic center of the lipase usually contains serine-histidine-aspartate/glutamate (Ser-His-Asp/Glu) (Derewenda *et al*., 1994). In the present study, lipases from *C. verticillata* and *G. candidum* were found to contain histidine, glutamic acid and aspartic acid. However, glycine and serine were not found, likely due to the lower amount of sample used and/or sensitivity of the HPLC used.

Oxyanion holes are composed of amino acids of the lipase active sites that contribute to stabilization of the catalytic reaction intermediates. Oxyanion holes are also involved in determining the specificity of lipase against substrates. For example, a GX type oxyanion hole usually hydrolyzes the substrate (medium/long carbon chain), while the GGGX type is found in lipases of shorter length. In the GX type, X represents either serine or a threonine, and in most cases aspartic acid or asparagine is also reported, which stabilizes the oxyanion holes (Pleiss *et al*., 2000). These findings support the presence of aspartic acid in both lipases observed in the current study.

5. Conclusions

In this study, lipases from the oil pollutant isolates, *Cunninghamella verticillata* and *Geotrichum candidum* were produced and purified. Purification was performed using ion-

exchange and gel-filtration column chromatography techniques. Overall, we provide amino acid composition analyses of the lipases of *C. veticillata* and *G. candidum*, which will be useful to current and future biotechnological applications.

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