Lipase Induction in *Mucor hiemalis*

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The influence on lipase induction in *Mucor hiemalis* of different types of triglycerides containing mainly oleic acid (olive oil), erucic acid (mustard oil), or saturated fatty acids of 8 to 16 carbons (coconut oil) was studied. The fungus was grown in shake flasks in a fermentation medium containing peptone, minerals, and glucose or one of the oils as the carbon source. Maximum lipase was produced when the initial pH of the fermentation medium was kept at 4.0. Addition of Ca^{2+} to the medium did not increase lipase production. The optimum pH for activity of both the mycelial and extracellular lipases was found to be 7.0. The fungus produced a significant amount of lipase in the presence of glucose, but the lipase activity increased markedly when olive oil was added to the medium at the beginning of the fermentation. Addition of olive oil at a later stage did not induce as much enzyme. Studies with washed mycelia showed that a greater amount of lipase was released when olive oil was present than when glucose was present. Among the various types of triglycerides used as the carbon source, olive oil was found to be most effective in inducing the lipase. Olive oil and mustard oil fatty acids inhibited the lipase more than those of coconut oil. The lipase induced by a particular type of triglyceride did not seem to be specific for the same triglyceride, nor was it inhibited specifically by it. Irrespective of the triglyceride used in the fermentation medium, the lipase produced was most active against coconut oil triglyceride, and this specificity, as shown by lipase activities in an *n*-heptane system, was not found to be due to a better emulsification of this oil. The lipase of *M. hiemalis* can be considered to be both constitutive and inducible.

Increased production of extracellular lipase in cultures of microorganisms growing in the presence of triglycerides and other lipids has been reported by various workers (4-6, 7-9, 12). Tsujisaka et al. (11), based upon experiments on lipase production in the mycelia and the broth, observed that the enzyme was formed inducibly by *Geotrichum candidum* in the presence of oils or fatty acids. Sugiura et al. (10) reported that long-chain fatty acids generally had an effect on lipase production by *Candida parapsilosis* in both the cells and the broth. A study on the influence of olive oil in lipase production by *Rhizopus* species, *Penicillium roquefortii*, *Mucor* species, and *G. candidum* showed that the cellular activity in all cases increased, whereas extracellular enzyme activity increased in some cases and decreased in others (2). Investigations of the influence of different natural triglycerides on *Rhizopus* species showed that glycerides of oleic and erucic acids caused a greater increase in lipase activity in both the mycelia and the broth as compared with coconut oil (1). In a more recent work on some of the *Mucor* species (6a), it was found that olive, coconut, and mustard oils increased lipase production in both the mycelia and the broth; olive oil effected the

greatest increase in lipase activities in both the mycelia and the broth. The lipase induced by a particular type of triglyceride was found, however, not to be specific to the same triglyceride.

The present work deals with an investigation of the influence of different triglycerides on the amount produced and specificity of the mycelial and extracellular lipase of *Mucor hiemalis*.

MATERIALS AND METHODS

Culture and inoculum. *M. hiemalis* (P-348) was obtained from the Plant Pathology Department, Agricultural University, Faisalabad, Pakistan. This organism was grown as Sabouraud agar slants at 25°C. Inoculum was prepared by washing the freshly subcultured slants with sterilized distilled water, and the spore suspension thus obtained was centrifuged (Beckman model J2-21 centrifuge) at 3,000 rpm for 30 min. The supernatant was discarded, and the spores were suspended in sterilized distilled water to obtain a standard concentration of 1.2×10^7 spores per ml.

Fermentation medium. The fermentation medium was the same as that used previously (1) and contained the following (in grams per liter of distilled water): peptone (Difco Laboratories, Detroit, Mich.), 30; KH_2PO_4 , 2.0; KCl, 0.5; MgSO_4 , 0.5 (all from BDH Chemicals Ltd., Poole, England; analytical reagent grade). The pH was adjusted to 4.0, unless otherwise

mentioned. A total of 285 ml of this solution was added to a 1-liter Erlenmeyer flask and autoclaved at 15 lb/in² for 15 min. A 20% (wt/vol) solution of glucose or a 20% (wt/vol) emulsion of the oil (prepared by homogenizing it in a top-drive homogenizer for 10 min in 10% gum acacia) was autoclaved separately, and 15 ml of either was mixed with the medium to make a final concentration of 1%. In some experiments, CaCl₂ (E. Merck AG Darmstadt, Germany) was also added to the medium.

Fermentation method. To each 1-liter flask containing 300 ml of fermentation medium, 5 ml of the inoculum was added. The flasks were incubated in a Gallenkamp orbital incubator shaker at 25°C and 200 rpm. Mycelia from the culture samples, collected at regular intervals, were removed by filtration and washed with distilled water till free of the broth. These mycelia were then stirred with chilled acetone, chilled acetone-ether (1:1) and then ether to obtain dry mycelial powder and kept in a desiccator over P₂O₅. The filtered broth was defatted by shaking it in the cold with chilled *n*-hexane, and the residual glycerides or fatty acids were removed along with the solvent layer.

Lipase assay in aqueous system. A suitable volume of the defatted broth (usually 5 ml) or a suitable weight of dried and defatted mycelia (usually 100 mg) was mixed with 5 ml of water, and the pH was adjusted to 7.0 unless stated otherwise. Five ml of 0.1 M McIlvaine buffer (pH 7.0) and 5 ml of a 10% triglyceride emulsion, prepared in 10% gum acacia by homogenizing in a top-drive homogenizer for 10 min, were then added. The mixture was incubated for 1 h at 37°C in a shaking water bath, after which 10 ml of acetone-alcohol (1:1) was added; the resulting mixture was then titrated against 0.1 N NaOH using thymolphthalein indicator. Blanks, obtained with boiled enzyme samples, were subtracted, and the activities were determined in terms of micromoles of free fatty acids released. All the determinations were done in duplicate. A unit of lipase was defined as the amount of enzyme that would liberate 1 μ mol of free fatty acids per min from the substrate under the assay conditions described.

Inhibition studies. To 45 ml of the defatted broth at maximal activity during fermentation was added 5 ml of a 10% emulsion of the different oils to make the final concentration of the oil 1%, as in the fermentation medium. The samples were incubated in a Gallenkamp orbital incubator shaker at 25°C for 24 h, and the remaining lipase activity was assayed against the oil

in the presence of which the lipase had been produced. Percent loss in activity was determined by comparing the activity with that of the corresponding 0-h sample. All the determinations were done in duplicate.

Lipase assay in *n*-heptane system. The method for lipase assay in the *n*-heptane system was similar to that described before (3). A total of 100 mg of the defatted and P₂O₅-dried mycelia was charged into a 50-ml Quickfit stoppered flask. A 20- μ l amount of water was then added such that it did not touch the mycelia. A 2.5-ml portion of the 10% (wt/vol) solution of the substrate in redistilled *n*-heptane (dried over anhydrous sodium sulfate) was then added, and the mixture was incubated in a shaking water bath at 37°C for 2 h. The flasks were then cooled and mixed with 2.5 ml of *n*-heptane. The mixture was centrifuged at 3,000 rpm for 5 min. A 2.5-ml portion of the supernatant was mixed with 10 ml of alcohol and titrated against 0.1 N NaOH using thymolphthalein indicator. Blanks, obtained by taking 2.5 ml of *n*-heptane alone instead of the substrate solution, were subtracted from the test readings, and lipase activity was determined in terms of micromoles of free fatty acids released. All the assays were done in duplicate.

RESULTS

Before dealing with the influence of triglycerides on lipase production by *M. hiemalis*, studies were undertaken to establish standard conditions for lipase production and the optimum pH for lipase activity.

Effect of initial pH on lipase production. *M. hiemalis* was grown in the fermentation medium containing olive oil with initial pH ranging from 3.0 to 7.0. Lipase activities in the mycelia and the broth at different fermentation periods are given in Table 1, which shows that the best initial pH for lipase production in both the mycelia and the broth was 4.0. Maximal mycelial and broth activities under this condition were 66 U/g and 198 U/100 ml, respectively, when the medium pH had reached 8.2. In all subsequent experiments, therefore, the initial pH of the fermentation medium was maintained at 4.0.

Effect of Ca²⁺ on lipase production. Different amounts, ranging from 0.005 to 0.02 M, of Ca²⁺ were added to the fermentation medium

TABLE 1. Effect of initial pH on production of lipase by *M. hiemalis* in olive oil medium^a

Fermentation period (h)	Initial pH														
	3.0			4.0			5.0			6.0			7.0		
	Medium pH	Broth lipase	Mycelial lipase	Medium pH	Broth lipase	Mycelial lipase	Medium pH	Broth lipase	Mycelial lipase	Medium pH	Broth lipase	Mycelial lipase	Medium pH	Broth lipase	Mycelial lipase
68	5.2	116	33	6.0	142	53	7.2	132	43	7.8	86	30	8.1	70	20
100	6.0	123	56	7.2	172	66	7.9	162	59	8.3	116	50	8.5	75	36
124	6.6	146	50	8.2	198	63	8.3	182	53	8.8	149	46	8.8	110	27
148	6.8	135	46	8.3	192	56	8.4	165	46	9.0	129	43	9.0	99	20

^a The lipase activities are reported as units per 100 ml of broth or per gram of dried and defatted mycelia.

containing 1% olive oil, and lipase activities were determined in the broth at different intervals during fermentation. It was found that, with the addition of low amounts of Ca^{2+} (0.005 to 0.01 M), greater lipase activity appeared in the broth in the earlier stages of fermentation (95 h), but there was little or no increase in activity later on (Table 2). In the medium to which no Ca^{2+} was added, there was a relatively low broth lipase activity after 95 h, but after 143 h of fermentation, the highest level of 198 U/100 ml of broth was reached.

Effect of pH on lipase activity. The influence of pH on the activity of extracellular and mycelial lipases of *M. hiemalis* grown in glucose medium is shown in Fig. 1. The buffers used were McIlvaine (pH 5.5 to 7.5) and borate (pH 8.0 to 9.0). Both the extracellular and the mycelial lipases were found to be most active at pH 7.0. When the organism was grown in the me-

TABLE 2. Lipase activity produced by *M. hiemalis* when different concentrations of Ca^{2+} were added to olive oil medium

Fermentation period (h)	Lipase activity (U/100 ml of broth) at the following concn:				
	None	0.005 M Ca^{2+}	0.01 M Ca^{2+}	0.015 M Ca^{2+}	0.02 M Ca^{2+}
95	137	152	173	132	142
119	165	168	178	159	152
145	198	188	181	172	172
166	185	178	165	150	142

FIG. 1. Effect of pH on lipase activity of *M. hiemalis*. Symbols: ○, micromoles of fatty acids per 100 ml of broth; ●, micromoles of fatty acids per gram of dried and defatted mycelia.

dium containing olive oil in place of glucose as the carbon source, the pH optimum of both the mycelial and broth lipases did not change.

Effect of addition of olive oil during fermentation. *M. hiemalis* was grown in medium containing glucose, and 1% olive oil was added at different stages of fermentation. Lipase production was determined in both the mycelia and the broth (Fig. 2). A significant amount of lipase activity was measured in both the mycelia and the broth when *M. hiemalis* was grown in glucose medium. Lipase production, however, increased with the addition of olive oil. Addition of olive oil during the early part of the growth phase resulted in a greater increase in lipase activity in both the mycelia and the broth. Nearly a threefold increase in lipase activity in both the mycelia and the broth was apparent when the oil was added to the fermentation medium at the time of inoculation.

Lipase production by washed cells. *M. hiemalis* was grown for 72 h in glucose medium. The mycelia were then harvested and washed with distilled water until free of any last traces of the medium; as much moisture as possible was then removed from the mycelia by placing the mycelia within folds of filter paper. A 1-g

FIG. 2. Effect of addition of olive oil during fermentation on lipase production by *M. hiemalis*. Symbols: ○, oil added at the time of incubation; ●, oil added after 8 h of cultivation; □, oil added after 24 h of cultivation; ■, oil added after 48 h of cultivation; ∇, no addition of olive oil.

portion of these cells was suspended in 250 ml of fermentation medium containing glucose or olive oil as the carbon source and incubated at 25°C and 200 rpm. Samples were taken at regular intervals, and lipase activity in the filtered and defatted broth was determined. Figure 3 shows that after incubation for 6 h, about 25% more lipase was released by mycelia in olive oil medium as compared with that released in glucose medium.

Effect of different triglycerides on lipase production. *M. hiemalis* was grown in media containing each of the following carbon sources: glucose, and olive, coconut, and mustard oils. Lipase activities produced in the broth as well as in the mycelia were determined in each case using olive oil as substrate (Fig. 4). Although all the oils effected increases in the mycelial and broth lipase activities over those attained in the glucose medium, olive oil was the most effective in inducing enzyme formation. As compared with 78 U/100 ml of the broth and 17 U/g of the mycelia in the glucose medium, the lipase activities in the olive oil medium increased to 198 and 66 U, respectively. The greater production of lipase in the presence of olive oil cannot be attributed to increased growth of the fungus because the dry mycelial weights were quite similar in both cases. Table 3 shows the relationship between mycelial weights (when at maximal activities) and maximal mycelial and broth lipase activities in the different media. In the mustard oil medium, the enzyme induction was not as marked as in the case of olive oil medium. In the coconut oil medium, although a lower mycelial growth was obtained, both the broth and the mycelial lipase activities per gram of mycelia were significantly higher than those in the mustard oil medium.

To find out whether the lipase produced in the presence of a particular tryglyceride was

FIG. 3. Lipase production by washed cells of *M. hiemalis* with glucose and olive oil. Symbols: ○, olive oil medium; ●, glucose medium.

FIG. 4. Effect of different triglycerides on lipase production by *M. hiemalis*. Symbols: ○, olive oil medium; ●, mustard oil medium; □, coconut oil medium; ■, glucose medium.

TABLE 3. Relationships between mycelial weights and mycelial and broth lipase activities when *M. hiemalis* was grown in media containing glucose or different oils as the carbon source

Carbon source	Mycelial wt (g/100 ml of medium)	Mycelial lipase activity (U/g)	Broth lipase activity	
			U/100 ml of medium	U/g of mycelia
Glucose	0.800	17	78	97
Olive oil	0.862	66	198	217
Mustard oil	0.839	36	98	118
Coconut oil	0.615	43	90	146

specifically active against the same triglyceride, the mycelial and broth samples at their maximal levels of lipase activity were assayed against all the triglycerides used as carbon sources in the fermentation medium. Table 4 shows that, irrespective of the triglycerides used in the fermentation, the lipase produced was more active against coconut oil than against olive or mustard oil.

Lipase inhibition. The extent of fatty acid inhibition on *M. hiemalis* broth lipase was studied in the presence of the different oils used as carbon source in the fermentation medium. Table 5 shows the percent loss in lipase activity of

TABLE 4. Maximal mycelial and broth lipase activities when *M. hiemalis* was grown in the presence of glucose or different triglycerides^a

Substrate	Glucose medium		Olive oil medium		Coconut oil medium		Mustard oil medium	
	Broth	Mycelia	Broth	Mycelia	Broth	Mycelia	Broth	Mycelia
Olive oil	78	17	198	66	90	43	98	36
Coconut oil	86	20	228	70	100	50	107	43
Mustard oil	66	13	158	60	86	36	86	30

^a Lipase activities are given as units per 100 ml of broth or per gram of dried and defatted mycelia.

the *n*-hexane defatted broth samples of olive, coconut, and mustard oil media when each of the samples was incubated with each of these oils for 24 h at 25°C; the activities were determined against the oil in the presence of which the lipase had been produced. Without any triglyceride, the percent loss in activity was only 6, 5, and 5% for olive, coconut, and mustard oil broths, respectively. On incubation with triglycerides, much greater inactivation of the enzyme was observed, which can be assumed to be due to fatty acid inhibition. Maximum loss in activity was observed when different broth samples were incubated with olive oil; a loss of 71, 42, and 51% was evident for the olive, coconut, and mustard oil broth samples, respectively. The loss in activity in mustard oil was slightly less than that in olive oil, but coconut oil caused much less inactivation for all the broth samples.

Lipase activity in the *n*-heptane system. Lipase activity in the defatted and dried mycelia of *M. hiemalis* produced in the media containing olive, coconut, and mustard oils was determined in the *n*-heptane assay system against each of the triglycerides (Table 6). Although the mycelial lipase was generally found to be less active in the *n*-heptane system than in the aqueous system, the proportion of activity against olive, coconut, and mustard oils in the *n*-heptane system was similar to that found in the aqueous system (Table 4). Greatest activity was found in the mycelia grown in the presence of olive oil, and the lipase was most active against coconut oil, irrespective of the triglyceride used in the fermentation medium.

DISCUSSION

Among the various *Mucor* species screened for lipase production, *M. hiemalis* was found to be the most potent and was therefore selected for the study of some of the aspects of lipase induction by triglycerides. Contrary to most earlier investigations, this paper deals with the lipase activities of the mycelia as well as the broth, as this may provide a better picture of lipase induction patterns.

TABLE 5. Percentage of loss of *M. hiemalis* broth lipase activity^a

Incubating oil	Loss in activity (%)		
	Olive oil broth	Coconut oil broth	Mustard oil broth
None	6	5	5
Olive oil	71	42	51
Coconut oil	16	21	6
Mustard oil	68	37	43

^a *n*-Hexane defatted broth was incubated as such and with different oils for 24 h at 25°C; the remaining activity was determined against the triglycerides in the presence of which the lipase was produced.

Before undertaking such an investigation, experiments were performed to determine some of the conditions enhancing lipase production. Thus, it was found that an initial pH of 4.0 in the fermentation medium resulted in greater lipase activities in both the mycelia and the broth (Table 1). It has been reported that free fatty acids in the culture medium inhibit lipase activity (1, 9). The influence of the addition to the fermentation medium of Ca²⁺, which would react with free fatty acids produced as a result of hydrolysis of triglycerides to form insoluble salts, was therefore studied. The results (Table 2), however, show that whereas in the earlier period of fermentation, 0.005 to 0.01 M Ca²⁺ resulted in increased lipase activity in the broth, the broth lipase activity in the later periods was higher in the medium which contained no Ca²⁺. Lower levels of maximal lipase activities in the media containing Ca²⁺ seemed to be due to the lower mycelial growth which was evident. The effect of pH on the activity of the extracellular and mycelial lipases of *M. hiemalis* showed that both were most active at pH 7.0 (Fig. 1). Replacing glucose with olive oil in the culture medium did not change this pH optimum.

In the medium containing glucose, *M. hiemalis* was found to produce a significant amount of lipase in both the mycelia and the broth (Fig. 2). The activity, however, increased with the addition of olive oil.

TABLE 6. *Lipase activity of dried and defatted mycelia in n-heptane system using olive, coconut, or mustard oil as substrate*

Substrate	μmol of fatty acids released per g of mycelia		
	Olive oil mycelia	Mustard oil mycelia	Coconut oil mycelia
Olive oil	125	50	70
Coconut oil	140	60	80
Mustard oil	80	30	30

The earlier the addition of oil, the greater was the increase in lipase production. It seems that the presence of olive oil at the stage when the cells are actively growing causes a greater increase in lipase formation by the fungal cells. The washed cells, obtained after growth in the glucose medium, produced lipase activity in the broth soon after they were reincubated in the glucose or olive oil medium, which can be assumed to be due to further growth (which was observed) as well as to synthesis and release of the enzyme into the medium. Lipase activity in olive oil medium was, however, greater than that in glucose medium (Fig. 3), and this supports the above results.

To study the effect of different triglycerides on lipase formation by *M. hiemalis*, the fungus was grown in the medium containing triglycerides which contained mainly oleic acid (olive oil), erucic acid (mustard oil), and saturated fatty acids of 8 to 16 carbons (coconut oil). Figure 4 and Table 3 show that olive oil induced a greater amount of lipase as compared with the other oils; this was followed by coconut oil and then mustard oil. Similar results have already been obtained in the case of *Rhizopus* (1) and other *Mucor* species (6a). Table 4 shows that the lipase induced by a particular triglyceride was not specifically active against the same triglyceride. Irrespective of the triglycerides used in the fermentation medium, the lipase was found to be most active against coconut oil compared with the other oils. The nature of the triglycerides inducing the lipase, therefore, does not seem to influence the glyceride specificity of the lipase.

When the defatted broth samples of the media containing different triglycerides were incubated at 25°C for 24 h, only small losses in lipase activities were observed (Table 5). The losses, however, increased greatly when the same samples were incubated with the triglycerides; this can be assumed to be due to inhibition by the fatty acids produced as a result of hydrolysis of the triglycerides. Irrespective of the oil inducing

the lipase, olive oil and mustard oil hydrolysis products caused greater inhibition as compared with coconut oil hydrolysis products in all broth samples. The lipase induced in the presence of a particular triglyceride thus does not seem to be specifically inhibited by its hydrolysis products. The lesser inhibition due to coconut oil fatty acids might contribute towards the greater lipase activity against the triglycerides of this oil. The inhibition effect of the fatty acid hydrolysis products of the oils suggests that the actual amount of lipase produced by *M. hiemalis* in the presence of olive or mustard oil may be greater than the apparent values.

The study of lipase action in organic solvent systems where the substrate is in dissolved form and the enzyme is in the solid state has been shown to be advantageous for many reasons (3). Lipase activity in the defatted and dried mycelia was therefore studied in the *n*-heptane system to eliminate the influence of the physical nature of different triglycerides. As the oils form a true solution in *n*-heptane, the lipase specificity for a particular type of triglyceride would be likely to be genuine, and not dependent upon oil emulsification. The pattern of the results on lipase activity of the mycelia in the *n*-heptane system (Table 6) was quite similar to those in the aqueous system. Therefore, the results on lipase production and activity in the aqueous system can be assumed to be genuine.

It seems that the lipase of *M. hiemalis* is both constitutive and inducible. The results presented here should be useful in understanding the mechanism of lipase induction in *M. hiemalis* and also in increasing the enzyme yields.

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