Pak. J. Sci. Ind. Res. 2005 **48**(6) 402-406

Effects of Physical and Chemical Treatments on the Enzymatic Activities of Rice Bran Lipases

M. Zahurul Haque*[a], Nurul Absar[b] and M. A. Jalil[a]
[a]BCSIR Laboratories, Rajshahi-6206, Bangladesh
[b]Department of Biochemistry, University of Rajshahi, Bangladesh

(received May 10, 2004; revised September 1, 2005; accepted October 25, 2005)

Abstract. The lipolytic activities of lipases lip-I, lip-II and lip-III, purified from rice bran variety Paijam, were investigated after physical and chemical treatments. The purified lipases had pH optima of 7.3-8.0 and temperature optima of 34-40 °C. The results indicated that lip-I was more stable than lip-II and lip-III. The lipases isolated from rice bran, variety Paijam, belong to the category of alkaline lipases. Bile salts were found to be weak activators for the activation of lipases, while maximum activities were obtained with deoxycholate. The activities of rice bran lipases were enhanced by the presence of Ca^{++} upto certain concentrations, while EDTA application strongly inhibited the lipolytic activities. Rice bran lipases were more sensitive to the denaturing agent guanidine-HCl than urea. The presence of heavy metal ions, such as Cu^{++}, Hg^{++}, Zn^{++}, Fe^{++}, strongly inhibited activities of the lipases, while such metals as Ba^{++}, Mg^{++} and Mn^{++} slightly increased the activities of lipases.

Keywords: lipolytic activities, rice bran lipases, bile salts, olive oil, heavy metals, rice variety Paijam

Introduction

Rice bran is a valuable by-product of the rice milling industry. It is the most important part of the rice grain as it contains proteins, minerals and vitamins. It is also considered to be a good source of oil, containing 16-22% oil (Ali *et al.,* 1999). Its only utility in Bangladesh, however, is its input as an ingredient in the livestock feedstuffs (Ali *et al.,* 2000). Beriberi is a very common and fatal disease in Bangladesh, which is linked to B_1 deficiency in the diet. The poorer classes of Bangladesh are the main victims of the disease, who live on a diet, deficient in vitamin B_1. Rice bran is a rich source of anti-neuritic vitamin B_1. After extraction of the oil, the left-over bran cake may also be used to increase the fertility of soil, as it contains important ingredients such as nitrogen, phosphorous and sulphur.

Lipases are an important class of enzymes which play a vital role in the digestion of fats and oils present in food materials (Hussain *et al.,* 1995). Although known for a long time, rice bran lipases have not been the subject of much work as compared with the lipases from other sources such as pancreas, castor bean and wheat germ (Shastry and Rao, 1971). Much of the earlier work on this enzyme pertains to its inactivation in order to 'stabilize' the bran and oil (Aizono *et al*., 1976; Funatsu *et al.,* 1971). The lipase activity is known to persist in the whole rice grains (paddy) for as long as 15 years. Recently, some work has been conducted on the purification and properties of this enzyme (Absar *et al.,* 1999).

The present study reports the purification of lipases from rice bran, variety Paijam, grown in Bangladesh. The purified lipases were subjected to various physical and chemical treatments, and the effects of these treatments on the lipolytic activities were also investigated. The study was expected to yield the useful information regarding some of the physicochemical properties, such as pH optimum and pH stability, temperature optimum and thermostability, the stability of lipases towards denaturating agents, and the enzymic activities in the presence of metallic salts. The experimental findings are expected to be helpful in establishing the conditions for chemical modifications of the lipases and in understanding the relationship between their structure and function.

Materials and Methods

Chemicals used and purification of lipases. Rice bran of variety Paijam was procured from Noor-Habib Auto Rice Mills, Rajshahi. Chemicals used in the study were bile salts and urea (BDH), guanidine-HC1 (Bio-Rad Laboratories, Richmond, California, USA), and miscellanceous other reagents used were all of analytical reagent grade.

The rice bran lipases (lip-I, lip-II and lip-III) were purified, in biologically active form, by gel filtration of 85% ammonium sulphate-saturated crude extract on Sephadex G-75, followed

*Author for correspondence; E-mail: mdzahurulhaque@yahoo.com

by ion-exchange chromatography on DEAE-cellulose as described previously.

Determination of lipase activity. Lipase activity was determined by using olive oil as the substrate in accordance with the method of Sugihara *et al*. 1(990). One unit of lipase activity was defined as the activity that liberates one micromole of fatty acids under specified conditions (pH and temperature). Specific activity of lipase was defined as the enzyme unit per mg of protein.

Determination of pH optimum and pH stability of lipases. To study the effect of pH on enzyme activity, the lipase solutions (0.5-0.6%) were dialyzed against 50 mM phosphate buffers of different pH values for 24 h, with frequent changes of buffers. After the necessary adjustment of pH values, by the addition of 0.2 N HC1 or 0.2 N NaOH, the enzyme activities were assayed using olive oil as the substrate, at room temperature (25-28 °C). Stability of the lipases at various pH values was examined by incubating the lipase solutions in buffers of a range of pH values from 1.5-10.5 at 10 °C for 24 h and then assaying their activities at room temperature, using olive oil as the substrate.

Determination of optimum temperature and thermostability of lipases. In order to determine the optimum temperature for lipase activity, the rice bran lipase solutions (0.5%) in 50 mM phosphate buffer (pH 7.5) were incubated at 10-60 °C for 30 min in a temperature controlled waterbath. The enzyme activities were assayed after cooling the heated lipase solutions in an ice-bath, using olive oil as the substrate. For examining the thermostability, the lipase solutions were incubated at various temperatures (10-60 °C) for 15 min at pH 7.5. After cooling the heated lipase solutions in an ice-bath, the enzyme activities were assayed at room temperature, as before.

Effect of bile salts. Bile salts, like tauroglycocholate, taurocholate, glycocholate and deoxycholate (1-2 mg/ml), were added to the reaction mixture containing 250 µl lipase solutions (0.3 mg/ml) and olive oil. The mixtures were gently shaken for a few min at 35 °C and then the lipase activities were determined.

Effect of calcium. Lipase solutions (0.3 mg/ml) in 10 mM Tris-HCl buffer, pH 8.0, were dialyzed separately against deionized water at 4 °C for 48 h, before incubation. The dialyzed lipase solution (200 µl) was incubated with $CaC1_2$ (0.001-0.5 M) for 30 min at 35 °C, using olive oil as the substrate. The lipase activities were assayed at room temperature.

Treatment with urea solution. To the lipase solutions (0.24 mg/ml) in 10 mM Tris- HC1 buffer, pH 8.0, were added different concentrations of urea (1, 2, 4 and 6 M). After incubation of the reaction mixture containing the enzyme solution and the substrate for 1 h at 35 °C, the lipase activities were assayed.

Treatment with ethylene diaminetetra acetate (EDTA). EDTA (0.001M-0.5 M) was added to the predialyzed lipase solutions (200 µl each) and incubated with substrate for 30 min at 35 °C accompanied by gentle stirring. The lipase activities were then assayed.

Treatment with guanidine-HCl. To the lipase solutions (0.3 mg/ml) in 10 mM Tris-HCl buffer, pH 8.0, was added guanidine-HCl at the concentrations of 0.5, 1.0, 1.5, and 2.0 M. The mixtures were incubated with olive oil for 1 h at 35 °C and then the enzyme activities were assayed.

Effect of mercuric chloride. To study the effect of mercuric chloride, the lipase solutions (0.25-0.3 mg/ml) were incubated with mercuric chloride at 0.001-0.002 M and the substrate for 30 min at 35 °C. The lipase activities were then assayed.

Treatment with various salts. The effect of some metallic salts on the rice bran lipase activity was determined by preincubating lipase solutions (0.25-0.3 mg/ml) at 0.001-0.002 M concentration of the reagent for 15 min at 35 °C. Then the substrate was added to start the reaction. After incubating for 30 min at 35 °C, the lipase activities were assayed.

Results and Discussion

pH optimum and pH stability of rice bran lipases. The lipolytic activities of the rice bran lipases at various pH values, from 3.0 to 11.0, are presented in Fig. 1. The activities of the lipases were greatly influenced by pH changes. The various lipases, lip-I, lip-II and lip-III showed maximum activities at the pH values of 8.0, 7.3-7.4, and 7.7-7.9, respectively. Beyond these pH values, both at the acidic and alkaline sides, the activities of all the lipases decreased abruptly. Thus, the optimum pH values calculated for the different lipases ranged between 7.3-8.0. The pH stability of the different rice bran lipases are shown in Fig. 2. The lip-I, lip-II and lip-III were found to be stable at the pH values between 4.0-8.0, 5.5-8.0, and 5.0-9.0, respectively.

It is evident from the results obtained that the optimum pH of the rice bran lipases ranged between 7.3-8.0. These results showed that lip-I was more stable in the acidic pH region, than the other two lipases. It may be concluded from these observations that the lipases isolated from rice bran, variety Paijam, belong to the category of alkaline lipases, similar to the lipases in peanuts (Sanders and Pattee, 1975), and unlike the lipases in castor bean (Ory *et al*., 1962).

Optimum temperature and thermal stability of rice bran lipases. The activities of rice bran lipases were determined on incubation at various temperatures and the results obtained are presented in Fig. 3. The activities of the lipases increased gradually and the maximum activities were observed around the temperatures of 37, 34, and 40 °C for lip-I, lip-II and lip-III, respectively. With further rise of temperature, the activities decreased abruptly and the enzymes lip-I, lip-II and lip-III lost almost 100% of their activities at the temperature ranges between 52-56 °C. To examine the thermal stability, the enzyme solutions were kept at various temperatures for 15 min and the activities were assayed after cooling at room temperature. As represented in Fig. 4, the enzymes lip-I, lip-II and lip-III were found to be stable up to 48, 40, and 44 °C, respectively. With regard to heat stability, lip-I was also found to be more stable than lip-II and lip-III. Rice bran lipases were found to be highly active in the temperature ranges between 34-40 °C. Lipases purified from other sources

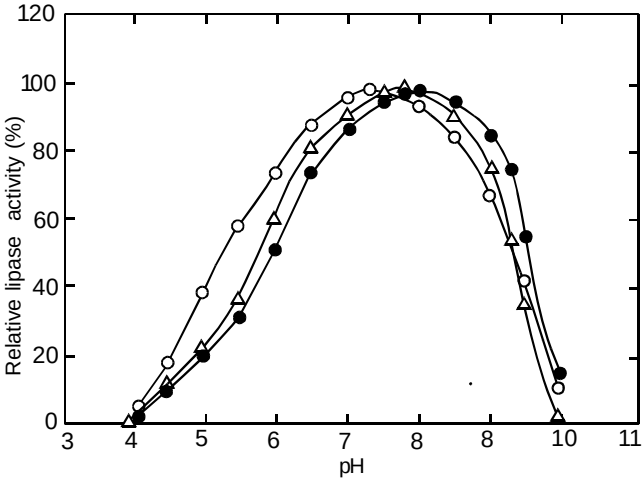

Fig. 1. Effect of pH on the activities of lipases, purified from rice bran, variety Paijam; substrate olive oil; the buffers used were: pH 4.0-5.5 (AcONa-CH_3COOH), pH 6.0-8.0 (NaH_2PO_4-Na_2HPO_4), pH 8.5-9.0 ($Na_2B_4O_7$-HC1), pH 9.5-10.0 ($Na_2B_4O_7$-Na_2CO_3); •–• lip-I; o – o lip-II; Δ – Δ lip-III; the highest activity was expressed as 100%.

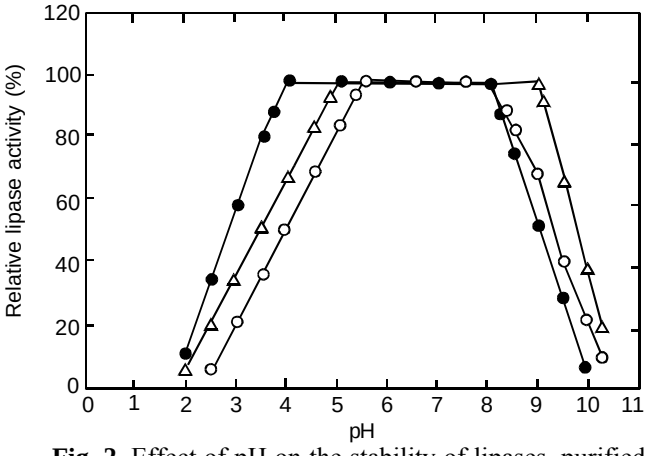

Fig. 2. Effect of pH on the stability of lipases, purified from rice bran, variety Paijam; substrate olive oil; the buffers used were: pH 3.0-4.0 (AcONa-HC1), pH 4.5-5.5 (AcONa-CH_3COOH), pH 6.0-8.0 (NaH_2PO_4-Na_2HPO_4), pH 8.5-9.0 ($Na_2B_4O_7$-HC1), pH 9.5-10.5 ($Na_2B_4O_7$-Na_2CO_3); •–• lip-I, o – o lip-II; and Δ – Δ lip-III; the highest activity was expressed as 100%.

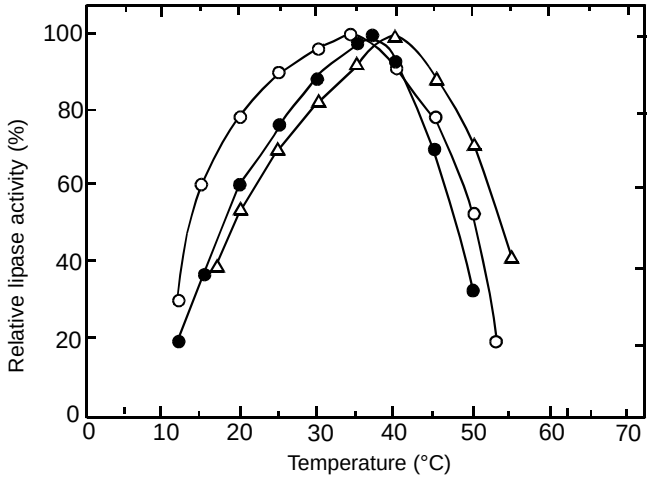

Fig. 3. Effect of temperature on the activities of lipases, purified from rice bran, variety Paijam; enzyme solutions in 50 mM phosphate buffer, pH 7.5, were incubated at various temperatures for 30 min; the highest activity was expressed as 100%; •–• lip-I; o – o lip-II; Δ – Δ lip -III.

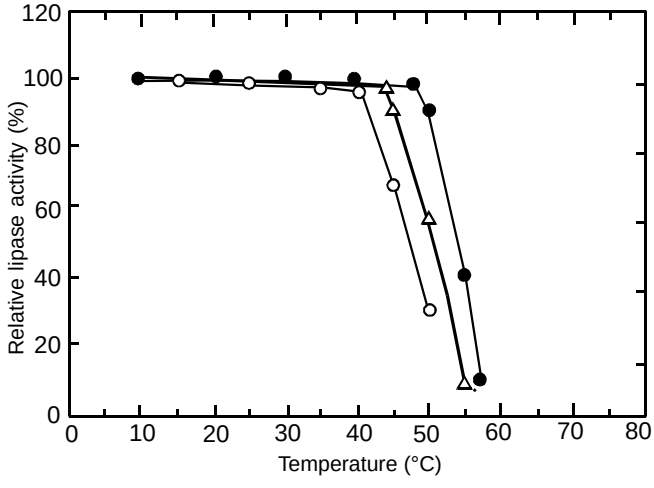

Fig. 4. Effect of temperature on the stability of lipases, purified from rice bran, variety Paijam; enzyme solutions in 50 mM phosphate buffer, pH 7.5, were incubated at various temperatures for 15 min; the highest activity was expressed as 100%; •–• lip-I; o – o lip-II; Δ – Δ lip -III.

have also been reported to be highly active in the temperature ranges between 30 to 40 °C. The optimum temperature for bovine milk lipase has been reported to be 30 °C, while those of two lipases from *Penicillium cyclopium* (Twai *et al*., 1975) were reported as 35 and 40 °C, and those of the three lipases from *Rhizopus delemer* (Twai and Sujisaka, 1974) ranged between 30 and 35 °C.

Effect of bile salts. Using olive oil as the substrate, the effects of different bile salts on the rice bran lipase activity are shown in Table 1. It may be concluded from the observations obtained that deoxycholate was the most effective in increasing the activity of lipases, followed by taurocholate, tauroglycocholate and glycocholate. However, on varying the concentrations of bile salts from 1 mg/ml to 2 mg/ml, no appreciable effect on the lipolytic activities were noted.

Effect of calcium. The effect of calcium salt ($CaCl_2$) on the activities of the lipases studied are presented in Table 2. The activities of lipases were noted to enhance at very low concentration of calcium, while the activities decreased gradually with the increasing concentration of calcium. It was observed that the activities of lip-I, lip-II and lip-III began to decrease after 0.002 M concentrations of $CaCl_2$, while at 0.5 M concentration, the lipases were found to be active only to the extent of about 70%. The activities of all the lipases were found to increase normally in the presence of calcium ion, even at low concentrations (1 mM or less), but were inhibited at the higher concentrations, which was consistent with the results reported elsewhere (Shastry and Rao, 1971).

Effect of urea salt. The relative activities of the lipases after treatment with different concentrations of urea are shown in Table 3. It was observed that the activities of lipases decreased gradually with the increase of urea concentration. Furthermore, lip-III was found to be slightly more sensitive to urea as compared to the other two lipases, lip-I and lip-II. In the presence of higher concentration of urea, lipases retained only about 5-10% enzyme activities, when treated with 6 M urea.

Effect of EDTA. The relative activities of the different lipases in the presence of different concentrations of EDTA are presented in Table 4, from which it may be noted that the activities of lipases decseased gradually with the increase in EDTA concentration. The enzymes lost their activities almost completely in the presence of 0.5 M EDTA. In view of these results it may be concluded that metal ions are essential for the activities of lipases.

Effect of guanidine-HCl. As shown in Table 5, the activities of lipases decreased gradually with the treatment of higher concentration of guanidine-HCl. It was found that the lip-I and lip-II retained about 52.16% and 24.14% activi-

Table 1. Effect of bile salts on the enzymatic activities of lipases, purified from rice bran, variety Paijam

Bile salt	Relative activities (%)		
	lip-I	liP-II	lip-III
Control (no salt added)	100	100	100
Glycocholate (1.0 mg/l)	101.70	103.06	101.09
Glycocholate (2.0 mg/l)	103.05	104.38	102.73
Deoxycholoate (1.0 mg/l)	108.31	109.00	109.00
Deoxycholoate (2.0 mg/l)	109.70	109.84	109.91
Taurocholate (1.0 mg/l)	107.00	106.68	106.00
Taurocholate (2.0 mg/l)	107.34	106.96	107.11
Tauroglycocholate (1.0 mg/1)	104.00	103.70	102.80
Tauroglycocholate (2.0 mg/l)	105.00	104.10	103.19

Table 2. Effect of calcium salt ($CaCl_2$) on the enzymatic activities of different lipases, purified from rice bran, variety Paijam

$CaCl_2$ con (molar)	Relative activities (%)		
	lip-I	lip-II	lip-III
0	100	100	100
0.001	112.30	115.00	109.00
0.002	110.45	112.70	108.00
0.005	102.32	105.00	100.00
0.010	98.00	100.36	99.10
0.100	81.10	82.00	80.45
0.200	70.80	72.00	70.91

Table 3. Effect of urea salt on the enzymatic activities of different lipases, purified from rice bran, variety Paijam

Urea conc (molar)	Relative activities (%)		
	lip-I	lip-II	lip-III
0	100	100	100
1	89.06	89.00	85.28
2	78.00	77.11	67.36
4	50.43	47.45	32.54
6	10.00	9.18	5.68

Table 4. Effect of EDTA on the enzymatic activities of different lipases, purified from rice bran, variety Paijam

EDTA con (molar)	Relative activities (%)		
	lip-I	lip-II	lip-III
0	100	100	100
0.001	47.38	49.32	53.84
0.005	35.00	36.11	40.04
0.010	27.43	29.00	32.00
0.200	13.42	13.60	14.16
0.500	5.32	5.75	7.03

ties, respectively, while lip-III retained only 10% activity after treatment with 1 M guanidine-HC1. The activities of the lipases decreased further with further increase in guanidine-HC1 concentrations, and activities of lip-I, lip-II and lip-III were lost completely on treatment with 2.5 M, 2 M and 1.5 M guinidine-HCl, respectively.

Effect of various metallic salts. The effect of some metallic salts on the lipolylic activities of rice bran lipases was tested at the specified concentrations of the reagents and the results are presented in Table 6. It was found that the presence of metallic salts, such as Ba^{2+}, Mg^{2+} and Mn^{2+} generally enhanced the activities of lipases, while the enzyme activities decreased markedly in the presence of salts of Al^{3+}, Fe^{+2}, Na^{+}, Cu^{2+}, Zn^{2+}, Na^{1+}, Hg^{2+}, etc. For a comparison, with respect to the diminishing activity of lipases, the salts may be arranged in the following order: $CuSO_4 > ZnSO_4 > NaF > HgCl_2 > ZnCl_2 > FeSO_4 > CuCl_2 > AlCl_3$.

Table 5. Effect of guanidine-hydrochloride (Gn-HCl) on the enzymatic activities of different lipases, purified from rice bran, variety Paijam

Gn-HC1 conc (molar)	Relative activities (%)		
	lip-I	lip-II	lip-III
0	100	100	100
0.5	86.60	54.32	43.16
1.0	52.16	24.14	10.00
1.5	28.00	7.04	0
2.0	8.14	0	0
2.5	0	0	0

Table 6. Effect of various metallic salts on the enzymatic activities of different lipases, purified from rice bran, variety Paijam

Test salts added (molar)	Relative activities (%)		
	lip-I	lip-II	lip-III
Control (no salt added)	100	100	100
$BaC1_2$ (0.001)	111.04	110.51	112.42
$BaC1_2$ (0.002)	113.00	113.00	114.63
$MgC1_2$ (0.001)	109.28	108.00	110.25
$MgC1_2$ (0.002)	110.13	110.04	114.05
$MnC1_2$ (0.001)	100.00	102.14	102.75
$MnC1_2$ (0.002)	100.00	104.00	105.07
$A1C1_3$ (0.001)	92.16	94.40	91.73
$AlCl_3$ (0.002)	90.03	89.85	89.00
$CuC1_2$ (0.001)	83.75	81.66	80.70
$CuCl_2$ (0.002)	70.72	66.54	65.82
$ZnC1_2$ (0.001)	68.37	66.32	66.14
$ZnCl_2$ (0.002)	28.91	26.24	22.53
$HgC1_2$ (0.001)	43.86	45.00	45.00
$HgCl_2$ (0.002)	23.92	26.17	25.36
$FeSO_4$ (0.001)	50.80	48.30	48.20
$FeSo_4$ (0.002)	32.76	29.41	28.18
$CuSO_4$ (0.001)	22.81	26.00	25.00
$CuSo_4$ (0.002)	0	0	0
$ZnSO_4$ (0.001)	31.00	26.41	20.05
$ZnSo_4$ (0.002)	7.73	5.80	0
NaF (0.001)	32.11	26.08	25.00
NaF (0.002)	10.31	12.25	8.00

Acknowledgement

We wish to express our thanks to Dr. Golbar Hussain, Director, BCSIR Laboratories, Rajshahi, Bangladesh for providing laboratory facilities to carry out the research.

References

Absar, N., Ali, M.M., Shahjahan, M. 1999. A comparative study on the purification and characteristic properties of lipases from five different varieties of rice brans. In: *Proc. 11th, Asian Agric. Symp. on Biological Diversity,* pp.1-10, Kumamoto, Japan.

Aizono, Y., Funatsu, M., Fujiki, Y., Watanabe, M. 1976. Biochemical studies on rice bran lipase. Part-2. Chemical properties. *Agric. Biol. Chem.* **40:** 317-324.

Ali, M.M., Absar, N., Hussain, M.G., Chowdhury, F.K.N., Ahsan, M.A., Jalil, M.A. 2000. Nutritional aspects of rice bran. Part-1. Amino acid content of rice bran. *Bangladesh J. Sci. Ind. Res.*, **35:** 1-4.

Ali, M.M., Absar, N., Hussain, M.G., Chowdhury, F.K.N., Ahsan, M.A., Jalil, M.A. 1999. Studies on rice bran oil. *Bangladesh J. Sci. Ind. Res.* **34:** 459-465.

Funatsu, M., Aizono, Y., Hayashi, K., Watanabe, M., Eto, M. 1971. Biochemical studies on rice bran lipase. Part-1. Purification and physical properties. *Agric. Biol. Chem.* **35:** 734-742.

Hussain, M.G., Ali, M.M., Chowdhury, F.K.N., Rahman, M. L., Khaleque, M.A. 1995. Investigation of rice bran and its oil. *Bangladesh J. Sci. Ind. Res*. **30:** 43-50.

Ory, R.L., Angelo, A.J., Altschul, A.M. 1962. The acid lipase of the castor bean: properties and substrate specificity. *J. Lipid Res.* **3:** 99-105.

Sanders, T.H., Pattee, H.E. 1975. Peanut alkaline lipase. *Lipids* **10:** 50-54.

Shastry, B.S., Rao, M.R.R. 1971. Studies on rice bran lipase. *Indian J. Biochem. Biophys.* **8:** 327-332.

Sugihara, A., Shimada, Y., Tominaga, Y. 1990. Separation and characterization of two molecular forms of *Geotrichum candidum* lipase. *J. Biol. Chem.* **107:** 426-430.

Twai, M., Okumura, S., Tsujisaka, Y. 1975. The comparison of the properties of two lipases from *Penicillium cyclopium*. *Agric. Biol. Chem.* **39:** 1063-1070.

Twai, M., Sujisaka, T. 1974. Purification, crystalisation and some properties of lipase from *Geotrichum candidum* Link. *Agric. Biol. Chem.* **38:** 1241-1247.