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Studies on the Chemical Modification of Rice Bran Lipase-1

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Abstract. Rice bran lipase-1 (lip-1) was treated with various groups of specific amino acids modifying reagents. Modification of arginine and histidine residues did not affect the lipolytic activities of the lipases. Acetylation of the lipases with acetic anhydride led to a complete loss of their lipolytic properties. However, citraconylation did not affect the lipolytic properties indicating the involvement of tyrosine residues at or near the active site of lipases. Acetylation of tyrosyl groups with N-acetylimidazole strongly reduced the lipolytic activities of lipases and the loss in activities was restored on deacetylation of tyrosyl groups. Modification of serine residues with diisopropyl fluorophosphate (DFP) inactivated the lipases completely, while the lipolytic activities of the lipases were reduced by about 50% after modification of cysteine residues with 5,5´-dithio-bis-2-nitrobenzoic acid (DTNB). Oxidation of lip-1 by N-bromosuccinimide (NBS) destroyed its activity completely, indicating the involvement of tryptophan residues at the active site. It is suggested from the results presented in this study that tyrosine, tryptophan and serine residues are located at or near the active site of lip-1.

Keywords: *Oryza sativa,* rice bran lipse-1, amino acids, acetylation, lipase modification

Introduction

Lipases are enzymes that catalyze the hydrolysis of triacylglycerols of animal fats and vegetable oils. These are recognized as being industrially important enzymes (Buhler and Wandrey, 1987; Macrae and Hammond, 1985). These enzymes are able to hydrolyze acylglycerides in aqueous medium and also catalyze the synthesis of esters in organic medium (John and Abraham, 1990). Further, these enzymes, especially those from microorganisms, have received increased attention after they were shown to be active even in nearly anhydrous water immiscible organic solvents (Zaks and Klibanov, 1984).

Chemical modification techniques have been used to study the amino acid residues involved in the active site of the enzyme molecules. Identification of these amino acid residues is a necessary requirement for understanding the lipase structure-function relationship. Many investigators have obtained much information about the catalytic site residues of some hydrolases, such as proteinase (Kaneda and Tominaga, 1975), and carboxypeptidases (Carey and Wells, 1972). Few reports have also been published on the active sites of lipases. With respect to porcine pancreatic lipase alone, it has been confirmed that the enzyme was a serine-histidine enzyme on the basis of the observations of complete inactivation by both paraoxon and photooxidation with methylene blue (De Caro *et al.,* 1981; Maylie *et al.,* 1972). In contrast to the configuration in animal tissues, the active sites of the lipases from higher plants have not yet been studied in detail, except for the observations by Ory *et al*. (1960) that sulfhydryl group was a part of the active system of castor bean lipases.

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Rice bran contains several types of lipases. Basic proteins of these lipases have been purified from rice bran of japonica variety by Aizono *et al*. (1973) and from indica variety by Sastry and Rao (1971). We have previously reported the purification and characterization of lipase-1 (mol wt 41000) from the rice bran of BR-11 variety of Bangladesh origin (Absar *et al*., 1999). It is a glycoprotein in nature (carbohydrate 7% and lipid 0.93%), containing high amounts of alanine, lysine, proline, serine, threonine, tryptophan and tyrosine.

The identification of specific amino acid residues within the active sites of lipases is important for understanding the relationship between structure and biological activity. The present investigations were undertaken to examine the active amino groups of lipase-1 (lip-1), after modifications with different group-specific modifying reagents, which are reported here.

Materials and Methods

Rice bran and chemicals used. Rice bran of BR-11 variety was purchased from the local auto-rice mills and the paddy growers authenticated the variety. Lipase-1 (lip-1) from this variety was purified in biologically active form and characterized as reported earlier (Absar *et al*., 1999). N-acetylimidazole, N-bromosuccinimide (NBS), diethyl pyrocarbonate (DEPC), citraconic anhydride and *p*-nitrophenylglyoxal were purchased from Fluka Biochemica, Switzerland. Sephadex G-25 was obtained from Pharmacia Fine Chemicals, Upsala, Sweden. Acetic anhydride and hydroxylamine were the products of British Drug House (BDH). All other reagents used, such as iodine, hydrogen peroxide, methylene blue and rose bengal were of analytical grade.

Lipolytic activity. The lipolytic activities of native and modified lip-1 were determined at room temperature according to the method as described by Sugihara *et al*. (1990). All the samples were dialyzed against distilled water and 50 mM phosphate buffer, pH 7.6, before the assay.

Treatment with some group-specific reagents. About 0.5 ml (= 0.1 mg) of the enzyme was incubated for 20 min at pH 7.0 with some specific-group reagents, such as iodine, hydrogen peroxide, methylene blue and rose bengal so as to make the final concentration of 1 mM. The enzyme activity was assayed by using olive oil as the substrate. The incubation period was 30 min in the case of periodate. The photooxidation with methylene blue and rose bengal, at pH 7.0, was carried out according to the procedure as described by Ray (1967). The lipase solution, 3 ml containing 2.5-3.0 mg protein, was transferred to a small beaker and placed under light with the light source (200 W) positioned 8 cm above the surface of the solution. Methylene blue or rose bengal solution (final conc 1 mM) was added to the lipase solution and exposed to light for 15 min, under constant slow stirring. The reaction mixture and the control (without treatment) were dialyzed against 50 mM phosphate buffer, pH 7.6, and the lipase activities were measured using olive oil as the substrate.

Reaction of lipases with diisopropyl fluorophosphate (DFP). The lipase solution (0.5-1 mg/ml in 50 mM phosphate buffer, pH 7.6) was mixed separately with 1 mM, 5 mM and 10 mM of DFP, and incubated at room temperature for 90 min. The lipolytic activities of treated samples and the control (without DFP) were measured as described earlier by using olive oil as the substrate.

Reaction of lipases with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB). The lipase solution (1 ml containing 0.65 mg) in 50 mM phosphate buffer, pH 7.0, was separately mixed with 1 mM and 10 mM of DTNB solution and incubated for 20 min at room temperature. The lipolytic activities of the control (without DTNB) and the treated samples were assayed after dialysis against buffer using olive oil as the substrate.

Acylation with citraconic anhydride. Amino groups were modified with citraconic anhydride as described by Habeeb *et al.* (1958). The enzyme solution, 2 ml (0.6-1.0 mg/ml) in 0.5 ml of 20 mM Tris-HCl buffer, pH 8.4, was treated with approx 400-folds molar excess of the reagent at 4 °C for 1 h. The activity of the modified lip-1 was assayed in the same manner as described before.

Modification of arginine residues. Arginine residues of lip-1 were modified following the reaction with *p*-nitrophenylglyoxal by the method of Bryan *et al.* (1981). To the 1 mg/ml enzyme solution in 0.1 M sodium pyrophosphate buffer, pH 9.0, was added 2.5 ml of 10% (v/v) *p*-nitrophenylglyoxal, and the mixture was incubated at 28 °C for 30 min. The extent of modification was determined as described by Habeeb *et al*. (1958), and the activity of modified lip-1 was assayed as described above.

Modification of histidine residues. Lip-1 was modified with diethyl pyrocarbonate (DEPC), essentially by the method of Miles (1977). The reaction was carried out at 20 °C in a quartz cuvette and pursued by difference spectroscopy. A baseline was first recorded by placing lipase solution in the buffer solution in the reference and sample cuvettes. To 2.5 ml lipase solution (0.6 mg/ml) in 20 mM sodium phosphate buffer, pH 7.2, in the sample cuvette, was added DEPC solution to make the final conc of 0.25 mM. At intervals of time, the difference in spectra were recorded against the lipase solution in the reference cuvette that had received the same volume of buffer solution instead of DEPC solution. The modification reaction was terminated when desired, by 100-times dilution of an aliquot of the reaction mixture with water. The number of histidine residues modified was calculated from the difference in molar extinction at 240 nm ($\Delta\varepsilon_{240}$=3,200/M/cm) for carbethoxy histidine (Miles, 1977).

Acetylation with acetic anhydride. Acetylation of lip-1 with acetic anhydride was performed as described by Fraenkal-Conrat (1959). While stirring continuously, 1 ml portions of acetic anhydride were added to 2.5 ml of lipase (0.8-1.0 mg/ml) in 50% saturated sodium acetate at 0 °C for 4 times, over the course of 1 h. The pH was maintained between 8 and 9 by the addition of 0.1 M NaOH. After 1 h, the treated sample was dialyzed against distilled water followed by dialysis against 50 mM phosphate buffer, pH 7.6, at 4 °C and the lipolytic activity of the modified sample was determined as described earlier. De-O-acetylation of acetylated lipase sample was made-up to 1 M with respect to hydroxylamine with the addition of an equal volume of 2 M hydroxylamine-HCl by keeping the pH to 7.5 with 0.1 M NaOH. The solution was mixed and left with occasional stirring for 15 min at 20 °C followed by exhaustive dialysis against distilled water at 0-4 °C. The total number of free amino groups in the native enzyme was estimated with reference to bovine serum albumin by trinitrobenzene sulfonic acid as described by Habeeb *et al*. (1958). The number of amino groups modified was determined by the estimation of remaining free amino groups with the same reagent.

Acetylation with N-acetylimidazole. The reaction was carried out at 20 °C as described by Riordan *et al*. (1965). To 2 ml of the lipase in 0.05 M sodium borate buffer, pH 7.5, was added approx 30- and 60-fold molar excess of N-acetylimidazole. The reaction mixture was kept at room temperature for 1 h. The number of O-acyl groups was determined from the change in absorbance accompanying the reaction. De-O-acetylation of the

acetylated lipases was done with l M hydroxylamine-HCl as described earlier in the case of acetic anhydride treatment. Before testing the lipolytic activity, the treated sample was dialyzed against 50 mM phosphate buffer, pH 7.6, at 4 °C for 12 h.

Modification of tryptophan residues with N-bromosuccinimide (NBS). The modification reaction was carried out according to the method of Spande and Witkop (1967). An aliquot (3.0 ml) of the enzyme solution (0.5 mg/ml) was placed in a quartz cuvette provided with a miniature stirring bar and absorption spectrum was recorded on a Shimadzu double beam spectrophotometer (UV-180). The experiment was conducted at 20 °C. Under stirring, 10 µl of NBS solution (2.4×10^{-3} M) was added and the absorption spectrum was measured after 2-3 min. To obtain the titration curve for the oxidation of tryptophan in lipase, NBS solution was added successively until the decrease in absorbance at 280 nm ceased. The number of tryptophan residues oxidized were calculated according to Spande and Witkop (1967).

Results and Discussion

As given in Table1, the lip-1 of BR-11 variety was very sensitive to the powerful oxidizing agents like iodine and hydrogen peroxide, as the lipolytic activity of lip-1 was destroyed completely in the presence of 1 mM iodine and hydrogen peroxide. From these results it can be assumed that tyrosine, methionine or cysteine residues were located at or near the active site. On the other hand, photooxidation of lip-1 with rose bengal and methylene blue did not affect the lipolytic activities suggesting that histidine and methionine residues were not located at the active site of lipases. Diisopropyl fluorophosphate (DFP) was found to be a powerful inhibitor, as it perfectly inhibited the lipase activity even at very low concentrations. The lipolytic activities of lip-1, purified from BR-11 variety of rice bran in the presence of various concentrations of DFP, are given in Table 1. The results indicated that the lipolytic activity was lost appreciably in the presence of 5 mM DFP, and was completely lost in the presence of 10 mM DFP. DFP at a concentration of 0.1 mM completely inhibited milk lipase after 15 min of incubation with trybutyrin as the substrate, whereas in the present case the activity of lip-1 was inhibited completely with 10 mM DFP after 90 min of incubation. The observations reported here strongly demonstrated that serine residues play an important role in the catalytic function of lip-1, which indicates that lip-1 is the serine enzyme like cotton seed (Ihle and Dure, 1972), carboxypeptidase from French beans (Carey and Wells, 1972), as well as a proteinase from melon fruit (Kaneda and Tominaga, 1975).

As presented in Table 1, the lipolytic activity of lip-1 was lost moderately after the treatment with 5,5´-dithio-bis-2-nitrobenzoic acid (DTNB) and more than 50% activity of lip-I was lost in the presence of 10 mM DTNB. In the present case, it was found that the activity of lip-1 decreased significantly with the modification of -SH group. From these observations it may be suggested that the -SH group of lip-1 may be located at or near the active site (Gargouri *et al*., 1988).

Table 1. The effect of some group-specific reagents on the lipolytic activity of the rice bran lipases, lip-1

Treatment reagent	Conc (mM)	Relative activity (%)	Type of group modified
Control		100	
Iodine	1	0	
Hydrogen peroxide	1	0	
Methylene blue	1	100	
Rose bengal	1	100	
DFP	1	26.00	
DFP	5	6.67	
DFP	10	0	
DTNB	1	84.25	
DTNB	10	50.00	
Citraconic anhydride	*	96.00	$-NH_2$
p-Nitrophenylglyoxal	**	98.14	arg
Diethyl pyrocarbonate	0.25	90.00	his

DFP=diisopropyl fluorophosphate; DTNB=5,5'-dithio-bis-2-nitrobenzoic acid; arg=arginine; his=histidine; */** refer to Materials and Methods sections: *acylation with citraconic anhydride; **modification of arginine residues

The effect of acetic anhydride on the lipolytic activity of lip-1 is given in Table 2, from which it is evident that the activity of lip-1 was lost completely after modification with acetic anhydride. The results also indicated that about five NH_2 groups and two tyrosine residues were associated with the activity of lip-1. Further de-O-acetylation of the modified lipase on treatment with l M hydroxylamine at pH 7.5, regenerated the tyrosine residues almost completely, while at the same time the lipolytic activity of the modified sample was also recovered to about 75% (Table 2).

Treatment of lipase with citraconic anhydride resulted in the binding of citraconyl groups to free amino groups (Habeeb *et al*., 1958). The effect of citraconylation on lipolytic activity of lip-1 is given in Table 1. From these results it is clear that lipolytic activity of the lipase was reduced slightly after citraconylation, suggesting that NH_2 groups may not be located at the active site.

Rice bran lipase was treated with *p*-nitrophenylglyoxal separately. Any modification did not produce any detectable change in the lipolytic activity of the lipases. This observation ruled

Table 2. The effect of acetylation on the lipolytic activity of rice bran lipase, lip-1

Treatment reagent	Group modified	Number of residues modified (mole/mole protein)	Relative activity (%)
Acetic anhydride	tyr*	2.1	zero
	$-NH_2$**	5.4	
Acetic anhydride followed by I M hydroxylamine, pH 7.5	tyr*	0.25	75
	$-NH_2$**	5.4	
N-acetylimidazole (15:1 molar ratio of N-acetylimidazole: protein)	tyr*	2.45	10
	$-NH_2$**	4.1	
N-acetylimidazole (30:1 molar ratio of N-acetylimidazole: protein)	tyr*	3.0	zero
	$-NH_2$**	5.2	
Acetylation followed by deacetylation with I M hydroxylamine, pH 7.5; ratio with protein @ 30:1	tyr*	1.05	75
None (control)	none	none	100

*number of residues of tyrosine as determined from the change in absorbance at 278 nm according to Riordan *et al.* (1965); **number of residues of $-NH_2$ as calculated by the method of Habeeb *et al.* (1958); tyr = tyrosine

out the possibility of arginine residues being involved in the enzymatic activity, as this reagent is specific for the modification of arginine.

The results of modification of lip-1 with N-acetylimidazole and its effects on the activity of the enzyme are presented in Table 2. It was found that three tyrosine residues and five $-NH_2$ groups were modified with N-acetylimidazole and the resultant modified lipase molecules lost lipolytic activity completely. From these results it can be concluded that tyrosine residues were located at or near the active site of lip-1, since the involvement of $-NH_2$ at the active site of lip-1 was ruled out from the results of citraconylation. This observation is confirmed further from the findings that deacetylation of O-acetyl tyrosine residue with NH_2OH at pH 7.5 regenerated about one tyrosine molecule and thus the modified lip-1 exhibited 75% recovery of its activity (Fraenkel-Conrat, 1959).

N-bromosuccinimide (NBS) can attack both tryptophan and tyrosine residues in the pH range of 4.0-4.5. In the present study, under the experimental conditions described, this reagent only modified tryptophan residues. This is borne out by absorption measurements, which clearly revealed that tyrosine residues were not modified because the isobestic point at 263 nm remained unchanged even after all the surface tryptophan residues had been modified. The results of NBS-oxidation of lip-1 at different pH values are presented in Table 3. It was found that the susceptibility of tryptophan residues toward NBS-oxidation was affected remarkably by pH. At pH 6.0, about two tryptophans were oxidized, while at pH 5.5, 4.5 and 4.0, about three, four and six tryptophan residues, respectively, were modified. As shown in Fig. 1, modification of first tryptophan residue lost about 50% lipolytic activity, while with the modification of the second tryptophan residue about 80% of the activity was lost. From these results it can be concluded that at least two tryptophan residues are located at or near the active site of lip-1 (Sankhavaram *et al*., 1984).

Table 3. The effect of pH on the oxidation of rice bran lipase, lip-1, on treatment with N-bromosuccinimide (NBS)

pH	Number of tryp residues modified (mole/mole protein)	Relative activity (%)
6.0	2.2	20
5.5	3.0	10
4.5	4.1	5
4.0	5.95	zero

tryp: tryptophon; the sample with no NBS added at pH 7.6 served as the control having 100% relative activity

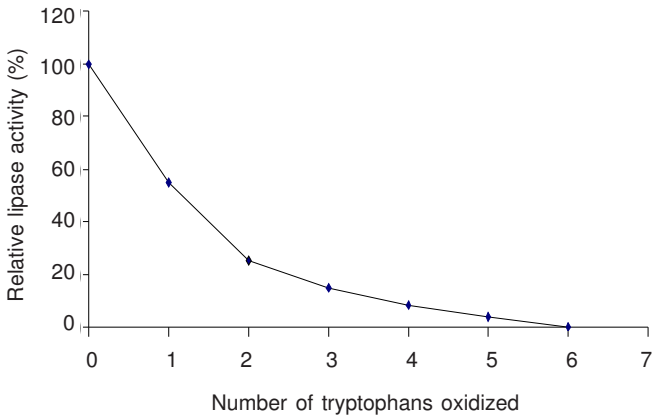

Fig. 1. Correlation between the loss of lipase activity against the increasing number of tryptophan residues modified by N-bromosuccinimide at pH 4.0.

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