CHEMICAL STUDIES ON RICE BRAN LIPASE

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ABSTRACT Cereal Chemistry 53(2): 190-200

Rice bran lipase contained 16.00 ± 0.15% nitrogen and was composed of about 320 amino acid residues. It also had a small quantity of lipid material (about 0.5%). The calculated molecular weight from SDS-polyacrylamide gel electrophoresis (and by gel filtration on Sephadex G-100) was about 40,000 ± 2,000. Disc gel electrophoresis in the presence or absence of 8*M* urea and results of experiments on molecular-weight determination indicated that the enzyme had no subunits. The amino terminal of the enzyme protein was blocked by acetyl group and the carboxy-terminal residue was phenylalanine. Among the sulfhydryl-blocking reagents, only *p*-chloromercuribenzoate significantly inhibited the enzyme activity. Iodine, hydrogen peroxide, copper sulfate, and higher concentrations of organofluorophosphates adversely affected lipase activity. Results of the experiments on the action of N-bromosuccinimide on lipase showed that the enzyme required one or more tryptophan residues for activity.

Rice bran lipase is an enzyme of economic significance (1–4), but interest in the enzyme has been recent. The enzyme has been purified to homogeneity by us (5) and by Funatsu *et al.* (6–8). The present communication describes the results of studies on some chemical properties of the enzyme. The results show that the enzyme described by Funatsu *et al.* (6–8) and that of the present communication differ although both are derived from rice bran.

MATERIALS AND METHODS

Materials

Rice bran from the local commercial variety of rice, Bangara sanna (*Oryzae sativa* var. indica S 1092), was obtained fresh from a local rice mill and the enzyme purified as described previously (5). The homogeneous enzyme was used in all studies. The following chemicals were purchased from the respective firms: N-bromosuccinimide (NBS), 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB), N-ethylmaleimide, iodoacetamide, 2-hydroxy-5-nitrobenzyl bromide (HNB), *o*-nitrophenylsulfenyl chloride (NPSC), 1-fluoro-2,4-dinitrobenzene, diisopropylphosphorofluoridate (DIPF), *o*-iodosobenzoate, and DNP-amino acids from the Sigma Chemical Company, rose bengal and methylene blue from the British Drug Houses, acrylamide, bis-acrylamide, and other chemicals required for gel electrophoresis from Eastman Organic Chemicals, Malathion from Cyanamide India, and silica gel for thin-layer chromatography (tlc) from the National Chemical Laboratory, Poona, India.

Methods

Protein concentration was determined by the method of Lowry *et al.* (9) using bovine serum albumin as the standard. Lipase activity was determined by the β-naphthyllaurate procedure as described previously (5).

Amino Acid Analysis

Amino acid analysis was carried out using the Beckman Model 120B analyzer essentially as described by Moore *et al.* (10). Enzyme protein (4 mg) was hydrolyzed in 5 ml 6*N* HCl for 24 hr in an evacuated sealed tube. It was then flash-evaporated at about 40° C repeatedly (five times) with water. The concentrate was dissolved in citrate buffer (pH 2.2) and used for analysis.

Methionine and histidine were estimated microbiologically using *Leuconostoc mesenteroides* P-60 (11). Cysteine content was estimated as described by Moore (12) after performic acid oxidation. The number of free sulfhydryl groups in the enzyme protein was determined by the Ellman method (13) using 5,5′-dithio-bis-2-nitrobenzoic acid. The spectrophotometric method of Goodwin and Morton (14) as modified by Edelhoch (15) was used to determine the tryptophan content. The ratio of tryptophan to tyrosine was calculated using the expression described by Paul *et al.* (16). Tryptophan content was also estimated microbiologically using *Streptococcus faecalis* (17).

For lipid determination, the enzyme protein (4 mg) was shaken at room temperature with a mixture of chloroform and methanol (2:1, v/v) and the lower solvent layer was concentrated and spotted on a silica gel tlc plate previously activated at 110° C for 1 hr. The plate was developed with hexane:diethyl ether:acetic acid (60:12:1, v/v) and the spots detected by exposing the plates to iodine vapors. This has been repeated on two to three different enzyme preparations.

Molecular Weight and Subunit Structure

The electrophoretic method of Weber and Osborn (18) was used to determine the subunit (peptide chain) molecular weight of the protein. Enzyme protein (2 mg in 0.5 ml) was treated with 1% sodium dodecyl sulfate (SDS) and 1% β-

mercaptoethanol for 2 hr at 36° C. Individual standard proteins were treated similarly in separate experiments. These were then dialyzed against 10 m*M* sodium phosphate buffer (pH 7.0) in the presence of 0.1% SDS and 0.1% β-mercaptoethanol. The samples were then electrophoresed at pH 7 for 4 hr using 10% gel containing 0.1% SDS. The gels were stained with Coomassie Brilliant Blue and destained with several changes of destaining solution. Electrophoresis was also performed at 4° C for 4 hr in the presence of 8*M* urea as described by Davis (19) and Reisfeld *et al.* (20); the sample was treated with 8*M* urea for 2 hr at 37° C before loading on gels containing 8*M* urea. The gels were stained with 1% amido black and destained with 7% acetic acid.

N-Terminal Analysis

The following procedure (21) was adopted to elucidate the blocked N-terminal residue. The enzyme protein (4 mg) was digested with pronase (1 mg/ml) at 37° C for 24 hr in the presence of 50 m*M* phosphate buffer (pH 7.0). This digest was applied to a Dowex-50 × 2 (hydrogen-form, 50–100 mesh) column (1.5 × 10 cm) which was then washed with water. The concentrated water eluate was placed on a Dowex-1 × 2 (chloride form, 200–400 mesh) column (1.5 × 10 cm). The acidic peptide was eluted with 0.03*N* HCl at 30 ml per hr and was detected by the ninhydrin reaction after alkaline hydrolysis of an aliquot as described by Hirs *et al.* (22). The ninhydrin-positive fractions were concentrated and converted into hydrazides by hydrazinolysis and to their DNP-derivatives as described by Phillips (23). The hydrazides were converted into DNP-derivatives by treating them in 0.2*M* sodium citrate buffer (pH 3.0) with 5% FDNB in ethanol. The reaction was allowed to proceed for 4 hr at 38° C. After evaporation of the reaction mixture *in vacuo* and addition of 3 ml water, the DNP-derivatives were extracted five to six times with 2–3 ml diethyl ether. The ether extract was dried and free dinitrophenol was removed by vacuum sublimation. The DNP-derivatives were characterized by paper chromatography using the solvent system 1-butanol saturated with 2*N* ammonia.

C-Terminal Analysis

The C-terminal residue was identified by the hydrazinolysis method (24). The hydrazides were converted by dinitrophenylation (24,25) to their DNP-derivatives and identified by paper chromatography. Enzyme protein (3 mg) was treated with 0.5 ml of fresh hydrazine hydrate (100%) at 100° C for 8 hr in a sealed tube. The hydrazinolisate was treated with benzaldehyde (0.5 ml) for 30 min, centrifuged, and the supernatant was lyophilized. It was then taken up in 3 ml water and treated with 0.3 g sodium bicarbonate and 6 ml 2.5% FDNB in ethanol for 4 hr. The mixture was then acidified with 2*N* HCl to pH 1 to 2 and the DNP-derivative extracted by ethyl acetate followed by ether. The combined solvent extracts were extracted twice with 10–15 ml 2% $NaHCO_3$. The bicarbonate extract was acidified to pH 1 to 2, extracted with ethyl acetate (10 ml × 3), and the combined solvent extract evaporated to dryness. The yellow extract was freed of dinitrophenol by sublimation and the DNP-amino acid was identified by paper chromatography using different solvent systems.

Photooxidation

Photooxidation with rose bengal at pH 7.0 and with methylene blue at pH 5.0

or 7.0 was carried out according to Westhead (26) and Ray (27), respectively. The protein solution (3 ml containing 3 mg protein) was placed in a small beaker and the light source (200 w) was positioned 8 cm above the surface of the solution. Rose bengal or methylene blue was added to the enzyme solution from 0.001 to 0.003%. The mixture was exposed to light for 15 min at 25° C and constantly stirred. The reaction mixture was subjected to photooxidation and the controls (without light and without the dyes) were dialyzed and tested for enzyme activity.

RESULTS AND DISCUSSION

The nitrogen content of higher plant lipases generally varies between 15.5 and 17.5%; that of rice bran lipase purified here was 16.0 ± 0.15% as determined by the micro-Kjeldahl procedure. Aizono *et al.* (7) reported 14.98% nitrogen in their rice bran lipase preparation.

Amino Acid Composition

The amino acid composition of rice bran lipase was determined as described under **Methods**. The results (averages from analyses of two different preparations) are tabulated in Table I. For comparison, the integral numbers of

TABLE I
Amino Acid Composition of Rice Bran Lipase

Amino Acids	Amino Acid per 0.312 mg Protein[a] μmol	Integral No. of Residues[b]		
		Calculated	This paper[c]	Aizono *et al.* (7)
Lysine	0.077	9.6	10	9
Histidine[d]	...	3.0	3	4
Arginine	0.076	9.6	10	11
Aspartic acid	0.401	50.1	50	34
Threonine	0.126	15.7	16	17
Serine	0.088	11.0	11	29
Glutamic acid	0.293	36.6	37	23
Proline	0.242	30.2	30	18
Glycine	0.313	39.1	39	44
Alanine	0.258	32.3	32	35
Half-cystine	0.110	13.7	14	7 (cys)
Methionine[d]	...	4.0	4	4
Valine	0.213	26.6	27	16
Isoleucine	0.057	7.1	7	7
Leucine	0.105	13.1	13	12
Tyrosine	0.038	4.7	5	17
Tryptophan[e]	...	4.0	4	9
Phenylalanine	0.062	7.5	8	16
Total	2.459	...	320	312

[a]Nitrogen content of similar aliquot was determined by the Kjeldahl procedure and multiplied by 6.25 to obtain the quantity of protein in the aliquot. Determined in the amino acid analyzer.
[b]Molecular weight 40,000 for both experiments.
[c]Enzyme isolated here.
[d]Determined microbiologically.
[e]Determined both microbiologically and spectrophotometrically.

residues as obtained by Aizono *et al.* (7) are also given. The total number of amino acid residues is approximately the same; 320 for the enzyme reported here and 312 for the Aizono preparation. There are significant differences between the two enzyme preparations with respect to certain amino acids. The contents of aspartic acid (+asparagine), serine, glutamic acid (+glutamine), proline, glycine, valine, and aromatic amino acids are noticeably different in each case. The recovery by amino acid analysis was reported to be about 87% by Aizono *et al.* (7), and it is around 83% here assuming a molecular weight of 40,000.

Evidence for the Presence of Lipid in Purified Lipase

The enzyme was found to contain triglycerides and free fatty acids (total approximately 0.5%) but not phosphoglycerides. The significance of these lipids in the enzyme is not clear. Okuda and Fujii (28) suggested that rat liver esterase was partly converted into liver lipase by forming a complex with lipid. Schoor and Melius (29) reported that porcine pancreatic lipase formed a complex with lipid and was consequently more stable. Whether any of these explanations holds in the present instance has to be ascertained.

Molecular Weight and Subunit Structure

Our previous report (5) stated that the enzyme had a molecular weight of about 41,000 ± 2,000, as determined by gel filtration. Its molecular weight was

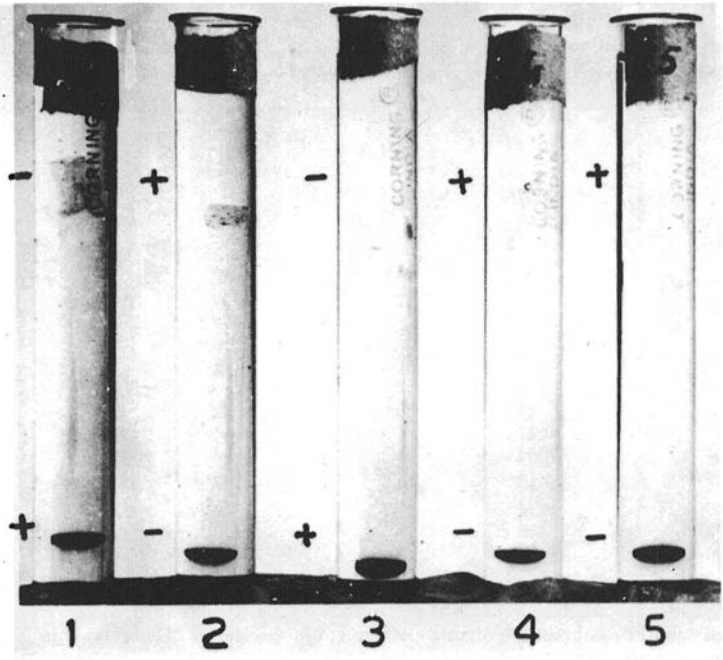

Fig. 1. Polyacrylamide gel electrophoresis pattern of rice bran lipase, using 200 μg of the enzyme protein, 6 mA/tube, 4 hr at 4° C. 1) pH 8.9, 2) pH 4.3, 3) enzyme and gel containing 8*M* urea at pH 8.9, 4) same as 3 at pH 4.3, and 5) same as 3 at pH 3.1.

estimated by the SDS-polyacrylamide procedure (18) using the following proteins as standards: bovine serum albumin (68,000), egg albumin (45,000), yeast alcohol dehydrogenase (37,500), and chymotrypsin (23,000). The average was about 40,000. If the enzyme had subunits, the molecular weight should have been lower than 40,000.

Supporting evidence for this conclusion is available from the disc gel electrophoresis experiments in the presence and absence of urea. The enzyme (100 μg) had the same mobility with or without urea (Fig. 1) and the densities of these protein bands as per microdensitometric tracings of the protein bands (run with and without 8*M* urea) were practically identical. Even when electrophoresis was conducted for shorter intervals of time (30 min or 1 hr) in the presence or absence of 8*M* urea, the enzyme mobility was the same and no faster moving components were observed. Thus, the rice bran lipase isolated here does not appear to contain any subunits and differs from that reported by Funatsu *et al.* (6–8).

N-Terminal Analysis

Attempts to elucidate the N-terminal group by Sanger's dinitrophenylation method (21) were not successful and only ϵ-DNP-lysine was obtained as a colored product in the aqueous extract. This suggested that the terminal α-amino group was blocked. The procedure described under **Methods** was adopted to characterize the blocked terminal residue. A schematic representation of the chromatogram is shown in Fig. 2a. The blocking group was identified as the acetyl group by the R_f value of its hydrazide and by the fact that the spot became brown when exposed to ammonia vapors. However, in addition to the brown spot, there were two other spots: one may be DNP-methionine and the other an unidentified spot with an R_f of 0.86. From the above experiment it was concluded that the N-terminal α-amino group was blocked by an acetyl group and either methionine or an unidentified residue was the acetylated N-terminal residue. This result also differs from that of Aizono *et al.* (7) who found that only glutamic acid was the N-terminal residue, although the enzyme was reported to have two subunits and two different C-terminal amino acids (see below).

C-Terminal Amino Acid

This amino acid was identified by hydrazinolysis of the enzyme using hydrazine hydrate (100%) as described under **Methods**. The developed chromatograms are graphically shown in Fig. 2 (bI and bII). The R_f value of the C-terminal residue coincided with that of phenylalanine. Hence it was concluded that phenylalanine was the C-terminal residue. Serine and glycine were reported by Aizono *et al.* (7) as the C-terminal residues. Whether these differences in subunit structure, and in N- and C-terminal amino acid residues, are due to varietal differences or other reasons is not clear.

Effect of Certain Group-Specific Reagents

The effect of certain group-specific reagents on enzyme activity was tested and the results are tabulated in Table II. DTNB at 1 m*M* had little or no effect on enzyme activity; even at 10 m*M*, there was only 20% inhibition. This is true for the other sulfhydryl-blocking reagents also, except pCMB. The free sulfhydryls of the enzyme are not readily available to these reagents and apparently pCMB

reaches them more readily than the others. When the enzyme was boiled, the sulfhydryls became easily available to DTNB. Four free sulfhydryls were found per enzyme molecule but, in the native enzyme, these were "buried" and not available to the Ellman reagent.

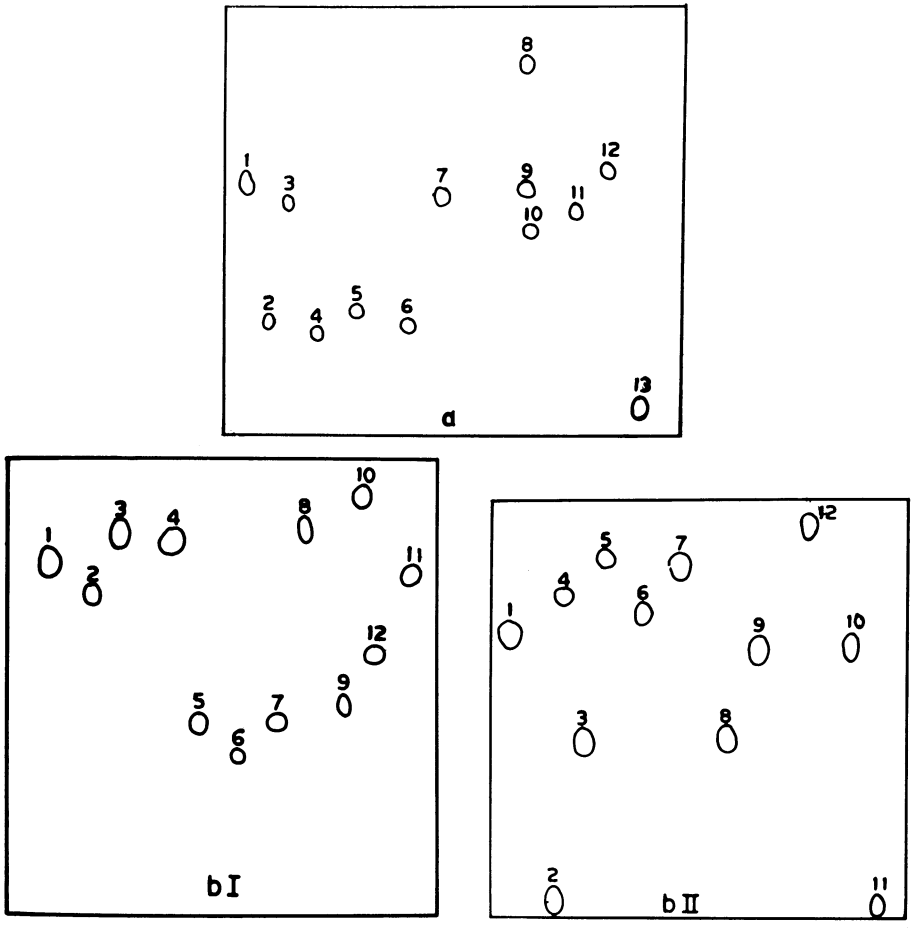

Fig. 2. Schematic representation of the paper chromatograms of DNP-derivatives of N- and C-terminal residues of enzyme protein, run 16 hr at 25° C. a) N-terminal analysis. Hydrazides: 1) DNP-leu, 2) ϵ-DNP-lys, 3) DNP-phe, 4) DNP-gly, 5) DNP-ser, 6) DNP-gln, 7) DNP-derivative of the acetyl hydrazide from ovalbumin, 8) unidentified DNP-derivative from the enzyme, 9) DNP-derivative of acyl hydrazide from enzyme (7 and 9 turn brown on exposure to ammonia vapors), 10) DNP-derivative from enzyme (DNP-met ?), 11) DNP-met, 12) Di-DNP-lys, and 13) DNP-glu. b) C-terminal analysis: I) t-amylalcohol saturated with phthalate buffer, pH 6.0. II) 1.5*M* phosphate buffer, pH 6.0. 1) DNP-met, 2) DNP-tyr, 3) DNP-phe, 4) DNP-val, 5) DNP-thr, 6) DNP-gln, 7) ϵ-DNP-lys, 8) DNP-enzyme sample, 9) DNP-arg, 10) DNP-leu, 11) DNP-lys, and 12) DNP-glu.

Cu^{2+} reacts with histidine and free amino groups of protein besides the sulfhydryl groups. The oxidizing agents, H_2O_2, iodine, and NBS, completely inhibited enzyme activity. The amino acids affected by these reagents were not examined in detail (except in the case of NBS; see below) by analysis for lack of

TABLE II
Effect of Certain Group-Specific Reagents on Lipase Activity[a]

Reagents	Final Concentration m*M*	Residual Activity %
Control	...	100
Iodoacetamide	1.0	100
	10.0	93
N-Ethylmaleimide	1.0	82
	10.0	78
DTNB	1.0	100
	10.0	80
pCMB	1.0	40
Copper sulfate	1.0	0
Hydrogen peroxide	1.0	0
Iodine	1.0	0
DFP	1.0	50
	5.0	0
Malathion	1.0	50
Methylene blue	0.001–0.003%	100
Rose bengal	0.001–0.003%	100
N-Bromosuccinimide	0.1	60
	1.0	0
2-Hydroxy-5-nitrobenzyl bromide	1.0	100
	10.0	60
o-nitrophenylsulfenyl chloride	1.0	0
Urea	1.5*M*	80
	8*M*	0
	8*M* Dialyzed	80
Guanidine HCl	0.3*M*	40
	2*M*	0

[a]Enzyme protein (0.1 mg in 0.5 ml of 50 m*M* phosphate buffer, pH 7.0) was incubated for 15 min with different reagents. Substrate (β-naphthyllaurate containing the reagent at the same concentration) was added to initiate the reaction. The preincubation period was 90 min for DIPF and Malathione and 30 min for periodate. Photooxidation was carried out as described under **Methods**.

an adequate quantity of the enzyme. The organofluorophosphates, DIPF and Malathion, also adversely affected enzyme activity. Apparently the serine (threonine?) residues proximal to or part of the active sites are blocked. Photooxidation in the presence of rose bengal or methylene blue did not affect lipase activity significantly. Under the same conditions, histidine and some tryptophan residues of a fungal glucoamylase are apparently affected (our unpublished experiments). This might mean that under experimental conditions, histidine/tryptophan/cysteine residues were unaffected, or that they were not essential for activity. Further experiments were not conducted to check these possibilities.

The effect of NBS, NPSC, and HNB indicated that the tryptophanyl groups were affected. The latter two reagents are specific for tryptophan but NBS oxidizes sulfhydryls to disulfide. To ascertain whether the inactivating effect of NBS on enzyme activity was due to the oxidation of sulfhydryl groups or of tryptophan, the enzyme protein was treated with different concentrations of NBS, and the spectral changes and enzyme activity measured. Enzyme activity of an aliquot was estimated in the presence and absence of an excess of glutathione (10 m*M*) or dithiothreitol. The results are graphically presented in Fig. 3, A and

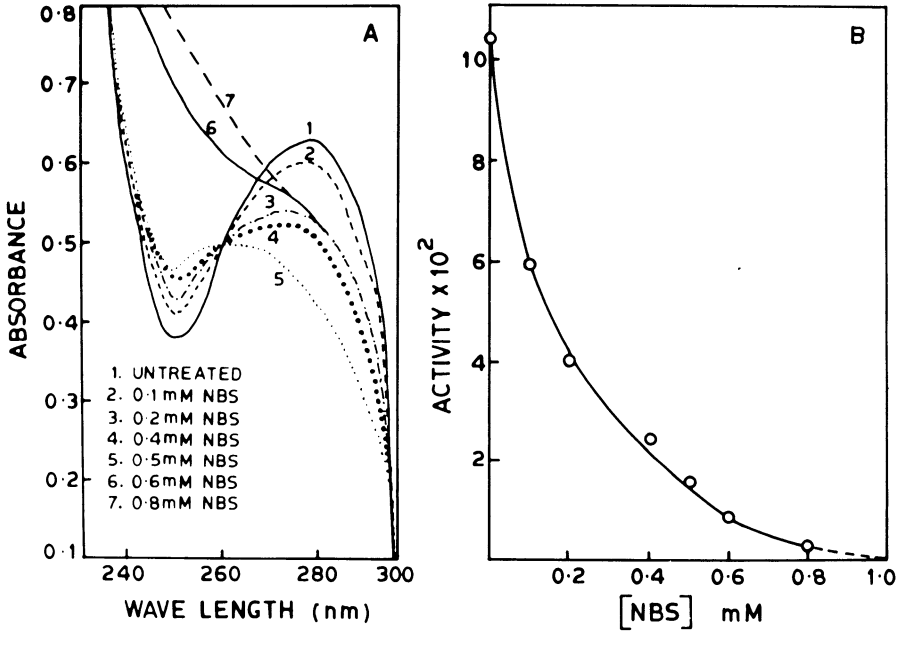

Fig. 3. Effect of NBS on A) the uv-absorption spectrum of the enzyme and B) enzymatic activity. The enzyme (400 μg/ml) in potassium phosphate buffer (50 m*M*, pH 7.0) was incubated with NBS at different concentrations for 15 min at 25° C. The absorption spectra of the control and the treated sample were taken. Reduced glutathione was added to the aliquots of the above to 10 m*M*, incubated for 15 min at 25° C, and the enzymatic activity was measured.

B. At lower concentrations (0.1 m*M*) of NBS, there was only a slight decrease in the absorbance of 278 nm but the activity was considerably decreased. As the concentration of reagent increased, there was a rapid fall in absorbance at 278 nm and activity was completely abolished. Incubation of the NBS-treated enzyme with glutathione or dithiothreitol did not restore enzyme activity, unlike the case of dihydrofolate (30). Although quantitation of the destruction of tryptophan by NBS was made, loss of the first two tryptophan residues apparently results in a rapid loss in enzyme activity. On the basis of these observations, it is tentatively concluded that the enzyme requires one or more tryptophan (or tyrosine) residues that are essential for its activity, in contrast to the lipases from pancreas (31,32), milk (33), liver (34), castor bean (35), and wheat germ (36), in which free SH groups or histidine residues were reported to be involved in activity. It is possible that such groups may be part of the active site or essential for maintaining the active conformation, such that destruction of the residues results in a loss in enzyme activity.

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