OXIDATIVE INACTIVATION OF *B. SUBTILIS* α-AMYLASE BY CHLOROPEROXIDASE*

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Chloroperoxidase and lactoperoxidase actively halogenate tyrosine residues of protein substrates and cause inactivation of α-amylases from *B. subtilis,* barley, hog pancreas and *Aspergillus oryzae.* Chlorotyrosine is produced in the inactivation of barley α-amylase. The *B. subtilis* enzyme is rapidly inactivated by chloroperoxidase in a process requiring either hydrogen peroxide, chloride and enzyme or sodium chlorite and enzyme. About 1.3 moles of peroxide is required per g-mole (48×10^3 g) for 95% inactivation of the dimeric enzyme. No chloride incorporation was found during inactivation of *B. subtilis* α-amylase. Amino acid analyses of *B. subtilis* α-amylase after acid and basic hydrolysis indicated methionine to be the only amino acid significantly modified as a result of the inactivation.

INTRODUCTION

Chemical modification is an important tool for studying structure-function relationships of enzymes and other biologically active proteins (1). The major problem encountered with this technique is the lack of specificity or selectivity of the chemical reagents. Since most reagents show reactivity toward more than one type of amino acid, the use of enzymes as modifying agents should bring about enhanced specificity in most cases. These specificities should be increased by steric limitations involved in bringing large proteins into close proximity for reaction. Therefore, enzyme modification techniques should offer significant advantages for studying enzyme functional groups.

Using chemical modification Connellan and Shaw (2) have implicated a tyrosine residue as being critical in *B. subtilis* α-amylase. This raises the possibility that a tyrosine residue may function as a critical amino acid in amylases. In order to investigate this assumption, two different approaches have been used:

1) A series of α-amylases have been subjected to treatments with lactoperoxidase and chloroperoxidase.

2) The inactivation of *B. subtilis* α-amylase by chloroperoxidase has been investigated in more detail. Both lactoperoxidase and chloroperoxidase inactivate all of the α-amylases studied, a result which is consistent with the occurence of critical tyrosine. However, the chloroperoxidase inactivation of *B. subtilis* α-amylase may not involve tyrosine. These results show the utility of enzymatic modification procedures with the peroxidases, but they also indicate the caution which must be used in interpreting results in terms of specific inactivation mechanisms.

MATERIALS AND METHODS

Lactoperoxidase from bovine milk was purchased from Sigma Chemicals (St. Louis), and chloroperoxidase was isolated from *Caldariomyces fumago* (3). *B. subtilis* type II-A, hog pancreatic and *Aspergillus oryzae* type IV-A α-amylases were purchased from Sigma Chemical. Barley α-amylase was prepared by the procedure of Mitchell (4).

Analytical Procedures

α-Amylase activity was measured using a modification of the procedure of Schuster and Gifford (5). One m*l* of 10 m*M* sodium acetate buffer, pH 4.8, with 0.01 *M* $CaCl_2$ is added to 1 m*l* of 1.4 mg/m*l* Merck solu-

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ble starch (special for diastatic determination) dissolved in the same buffer. A 10-μl quantity of enzyme solution is added, followed by appropriate buffer to make a 3-ml volume; the mixture is then incubated at 30 C for 1 min. and quenched with 1 ml 2.5 mM KI with 5 mM I_2 solution in 50 mM HCl. Before reading the absorbance at 620 nm, the assay solution is diluted with 5 ml H_2O. Amylase activity was also measured by the "Azure Amylose" procedure (6).

Protein concentrations were determined by the method of Lowry et al. (7). The activity of chloroperoxidase was measured using the activity assay of Morris and Hager (3) and was about 23.8×10^4 units/ml (1 unit = 1 μmole dichlorodimedone formed/min. at room temperature). Protein samples for amino acid analysis were hydrolyzed in 6 N HCl at 110 C for 24 hr. (8) or in 4 N NaOH at 100 C for 8 hr. (9). Analyses were performed on a Beckman 120 C amino acid analyzer.

Barley (4) and *B. subtilis* (10) α-amylase were judged to be homogeneous by gel electrophoresis and ultracentrifugation.

Lactoperoxidase Treatment of α-Amylases from *B. subtilis*, *Aspergillus oryzae*, Hog Pancreas and Barley

A 0.1-ml quantity of α-amylase solution (10.0 mg/ml) in 10 mM acetate buffer, pH 4.8, with 10 mM $CaCl_2$ was incubated with 0.2 ml of 5 mM KI, 10 μl of 0.01 mM H_2O_2, 5 μl lactoperoxidase solution (1 mg/ml) and 0.7 ml 50 mM acetate buffer, pH 5.0, at 37 C. Three blanks were used, one without lactoperoxidase, one without KI, and one without α-amylase. At 10-min. intervals 10-μl aliquots were removed for activity assay.

Chloroperoxidase Treatment of α-Amylase

The reaction mixture containing 1.0 mg of amylase, 10 mM in Ca $(NO_3)_2$, 60 mM in NaCl and 2 mM in H_2O_2 in 2.0 ml of 10 mM acetate buffer (pH 4.8) was incubated at 37 C with 5 μl of chloroperoxidase (1.2 mg/ml, 1220 units). Three blanks were used, one without chloroperoxidase, one without NaCl, and one without α-amylase. After 10-min incubation, 10-μl aliquots were removed for amylase activity assay.

RESULTS

Inactivation of Amylase with Lactoperoxidase and Chloroperoxidase

All the α-amylases investigated can be inactivated by either lactoperoxidase or chloroperoxidase (Table 1). The reactions require enzyme, peroxidase and halide. Deletion of any of these from the reaction mixture decreased or eliminated the inactivations. Some inactivation of the pancreatic and *A. oryzae* amylases is observed in the absence of the modifier enzymes, suggest-

TABLE 1. *Lactoperoxidase* and *chloroperoxidase treatment of α-amylase from barley, hog pancreas,* Aspergillus oryzae *and* B. subtilis. *Amylase activity determined by the "Azure Amylose" procedure (6).*

Protein	% Activity[a]	
	Lactoperoxidase Treatment	Chloroperoxidase Treatment
Barley α-Amylase		
Complete	35	30
Without enzyme	100	100
Hob Pancreatic α-Amylase		
Complete	0	—
Without enzyme	71	41
Aspergillus oryzae α-Amylase		
Complete	15	5
Without enzyme	85	100
Bacillus subtilis α-Amylase		
Complete	5	0
Without enzyme	100	100

[a]The relative activities were similar in four separate experiments.

ing an oxidative effect of peroxide alone. Neither the barley nor *B. subtilis* enzymes are inactivated under these conditions. Both enzymes show homogeneity as determined by gel electrophoresis and ultracentrifugation (4, 10). It is questionable whether these effects involve direct action on the enzymes, since the inactivated enzymes are less highly purified preparations than the other two and may contain other oxidizable substances. After 10 min, maximum inactivation had occurred; therefore, all peroxidase reactions were conducted with 10-min incubation. The *B. subtilis* inactivation with chloroperoxidase was essentially completed after 1 min.

The modification of tyrosine does occur under the conditions used, as shown by

TABLE 2. *Tyrosine and chlorotyrosine content after chloroperoxidase modification of barley α-amylase.*[a]

The complete reaction mixture consisted of 0.5 mg of protein, 10 mM sodium acetate buffer, pH 4.8, 60 μmoles NaCl, 5 μmoles H_2O_2, 1.2 μg (238) units) of chloroperoxidase in 1 m*l* of solution and was incubated at 37C for 7 minutes. The samples were then frozen, lyophilized and used directly for amino acid analysis (8).

Protein	Residue/1000 Residues	
	Tyrosine	Chlorotyrosine
Barley α-Amylase		
Complete	29	10[a]
Without chloroperoxidase	39	0
B. subtilis α-Amylase		
Complete	24	0
Without chloroperoxidase	24	0
Bovine Serum Albumin		
Complete	23	8
Without chloroperoxidase	34	0
Carboxymethyl Bovine Serum Albumin		
Complete	25	6
Without chloroperoxidase	34	0

[a]The results of 6 independent experiments.

TABLE 3. *Complete amino acid analysis of barley α-amylase treated with chloroperoxidase*

Amino Acid	Residues/10^3 residues	
	Control[a]	Chloroperoxidase-treated protein[b]
Aspartic acid	117	122
Threonine	41	50
Serine	41	46
Glutamic acid	104	115
Proline	85	80
Glycine	107	109
Alanine	93	98
Valine	73	77
Methionine	19	23
Isoleucine	41	34
Leucine	79	78
Tyrosine	39	29
Phenylalanine	39	40
Lysine	41	41
Histidine	31	36
Arginine	50	56
Tryptophan	9	9
Half Cysteine	24	22
Cysteic acid	27	—

[a]Three independent analyses.
[b]A single analysis.

amino acid analysis for chlorotyrosine of the barley α-amylase or bovine serum albumin (Table 2). Complete amino acid analysis of barley α-amylase that had been treated with chloroperoxidase showed no significant change in amino acid residues other than tyrosine (Table 3).

Inactivation of *B. subtilis* α-Amylase

Because the amylase from *B. subtilis* can be obtained in highly purified form in quantity, a more thorough examination of the inactivation of this enzyme was undertaken (see Figure 1). In the presence of chloroperoxidase and excess chloride, 1.0 mg of α–amylase is maximally inhibited by 0.05 μl of 0.5 m*M* H_2O_2. Thus the amount of peroxide required for 75% inactivation is slightly greater than one mole of peroxide per mole of enzyme protein, on the basis of a subunit molecular weight of 24×10^3 (10, 11). Figure 2 shows the inactivation of *B. subtilis* α-amylase to depend on the presence of chloride and chloroperoxidase.

Analysis of *B. subtilis* α-amylase for chlorotyrosine by amino acid analysis was negative (Table 2). Likewise, less than 0.1 mole of ^{36}Cl was incorporated per mole of *B. subtilis* amylase that was 93% inactivated, as measured by the starch-iodine amylase assay. These results suggest that the peroxidase is inhibiting amylase by oxidation rather than chlorination. Inactivated amylase was therefore analyzed for histidine and tyrosine by amino acid analysis and for tryptophan and tyrosine by the method of Edelhoch (12). As shown in Table 3 no loss of these amino acids was found in the inactivated enzyme. We also treated chloroperoxidase with *N*-acetyltryptophan, *N*-acetyltyrosine and *N*-acetylhistidine and observed no modification. This is consistent with the observation that there is no incorporation of radioactively labeled chloride and that spectrophotometric determination of tyrosine and tryptophan shows no differences between the chloroperoxidase-treated and untreated *B. subtilis* α-amylase.

Sulfur-containing amino acids are also susceptible to oxidative processes (16). *B. subtilis* α-amylase contains no free sulfhydryl or disulfide groups (13), but it does have methionine. Since methionine sulfoxide, the product of limited oxidation of this amino acid, may be converted back to methionine by acid hydrolysis, amino acids were analyzed after basis hydrolysis of different samples of native and inactivated enzymes. The native enzyme consistently showed 4-5 methionines per mole, while the inactivated enzyme showed only 2-3 residues/mole of protein (Table 4). These results indicate that 1-2 methionines

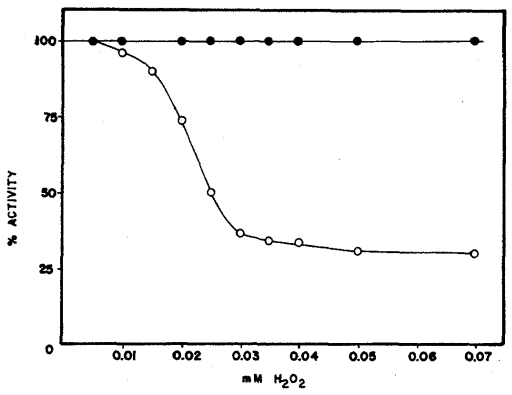

FIGURE 1. Inactivation of *B. subtilis* α-amylase by chloroperoxidase and variable concentration of H_2O_2. The incubation mixture contained 10 mM sodium acetate buffer pH 4.8, 60 mM NaCl (1.2 μg chloroperoxidase (288 units)) and 1.0 mg of *B. subtilis* α-amylase in 1.0 ml of solution. Amylase activity was determined by the "Azure Amylase" method (6). ●——● controls (no chloride); o——o complete system.

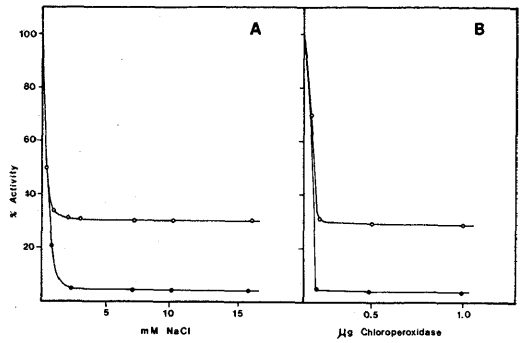

FIGURE 2. Chloride and Enzyme Dependence of Inactivation.

A) The incubation mixture contains 0.5 μmoles of H_2O_2, 10 μmoles sodium acetate, pH 4.8, 288 units of chloroperoxidase, 1.0 mg of *B. subtilis* α-amylase and varying amounts of NaCl in a 1.0 ml volume.

B) The incubation medium contained 1.0 mg of *B. subtilis* α-amylase, 0.5 μmole H_2O_2, 60 μmole NaCl and enzyme in 1.0 ml of a 10 ml sodium acetate buffer, pH 4.8. o——o α-Amylase activity determined by the "Azure Amylase" method (6). ●——● α-Amylase activity determined by the Modified Shuster and Gifford method (5).

are altered in the inactivated enzyme. This is the result of 8 independent amino acid analyses of separate experiments.

Another interesting facet of the inactivation is the chloride requirement (Table 5). This suggests that the peroxidase intermediate in the inactivation reaction is an oxidized form of chlorine rather than oxidized enzyme. Examination of the effects of various oxidized-chlorine salts shows that only $NaClO_2$ will substitute for H_2O_2 in the enzyme inactivation. This effect is consistent with studies showing that an oxygenated form of chloride is the active intermediate in the catalytic mechanism (7).

DISCUSSION

B. subtilis α-amylase is rapidly and specifically inactivated by treatment with chloroperoxidase in a process requiring either enzyme plus chloride and peroxide or enzyme plus chlorite. This inactivation does not occur with the production of chlorotyrosine as in the case of barley α-amylase, which has 24% of its tyrosine residues converted to chlorotyrosine. Since *B. subtilis* α-amylase tyrosine residues are not modified by chloroperoxidase and also since ^{36}Cl is not incorporated into the protein, then perhaps either tyrosine is oxidized or another residue necessary for catalysis or binding is inactivated. Our data suggest that another amino acid residue in *B. subtilis* α-amylase which may be involved catalytically is modified by chloroperoxidase.

Two factors suggest that the inactivation does not occur by direct action of the enzyme on a key catalytic residue. First, maximal inactivation requires only slightly more than stoichiometric quantities of peroxide per mole of enzyme, a molecular weight of 48×10^3 being assumed (10). Second, the sigmoidal nature of the inactivation curve indicates that most of the stoichiometric amount of peroxide can be utilized with virtually no inactivation of the enzyme. An alternative explanation of these results is that modification occurs at one or more residues of one or more of the protomers of the dimeric enzyme to cause a conformation change resulting ultimately in almost total loss of the enzyme activity. This explanation would account for the limited peroxide requirement and the sigmoidal nature of inactivation. Another interesting observation is that chloroper-

TABLE 4. *Amino acid analysis of* B. subtilis *α-amylase inactivated with chloroperoxidase.*

H_2O_2 Conc. (m*M*)	Activity (%)	Lys	His	Arg	Tyr	Phe	Met	Trp[a]	Tyr[a]
		(Residues/10^3 Residues)						(Residues/48×10^3 daltons)	
0	100	21.7	11.1	16.2	29.8	21.1	4.7[b]	14.6	19.8
0.05	3.4	21.9	11.1	17.1	29.1	21.3	2.8	15.0	20.0
0.10	2.1	22.6	11.3	17.2	28.5	20.8	2.5[c]	—	—

[a]Tryptophan and tyrosine were determined according to the method of Edelhoch (12).
[b]SD ± 0.9 from 8 experiments.
[c]SD ± 0.5 from 5 experiments.

TABLE 5. *Effect of salts of chlorine oxyacids on chloroperoxidase-treated* B. subtilis *α-amylase.*

The incubation mixture contained 10 mM sodium acetate buffer, pH 4.8, 1 mg α-amylase, 1.29 μg of chloroperoxidase (288 units) and 0.5 m mol of the appropriate salt in 1.0 m*l*. After a 30-minute incubation period at 30 C, 100 μ*l* was removed to measure α-amylase activity. ($NaClO_2$ alone (without chloroperoxidase) inhibited the enzyme to give 65% of the controls.) The "Azure Amylose" assay was used (6).

Additions	% Activity			
	NaClO	$NaClO_2$	$NaClO_3$	$NaClO_4$
	(0.5 m*M*)			
+ Chloroperoxidase	100	29[a]	100	100
— Chloroperoxidase	100	100	100	100

[a]SD ± 2.5 from 5 experiments.

oxidase inactivation is not reversed in the presence of substrate. That is, there is no protection by substrate. This also suggests that the inactivation occurs at noncatalytic residues. Methionine appears to be the amino acid modified, although we did not obtain a direct correlation of methionine oxidation and enzyme inactivation other than by amino acid analysis for methionine residues. We did not observe stoichiometric amount of methionine sulfoxide; however, amino acid analysis of chloroperoxidase-treated *B. subtilis* α-amylase showed a decrease in the number of methionine residues per 10^3 amino acid residues. Sulfur-containing amino acids interfere with the peroxide-catalyzed halogenation, iodide oxidation, and tyrosine halogenation (16). In the case of methionine the mechanism may involve a methionyl-sulfonium derivative.

Also notable is the relative stability of barley α-amylase enzymes. An even more interesting observation is the comparison of *A. oryzae* and *B. subtilis* α-amylase; both contain the same relative amount of methionine and are equally susceptible to chloroperoxidase, but they respond differently to lactoperoxidase. We might also add that the *A. oryzae* α-amylase is quite different from *B. subtilis* α-amylase inasmuch as *A. oryzae* enzyme contains sulfhydryl groups and a carbohydrate moiety (15) whereas *B. subtilis* α-amylase has neither cysteine (13) residues nor a carbohydrate moiety.

Although these peroxidases are often used for halogenation reactions, the inactivation of *B. subtilis* α-amylase apparently involves an oxidation process with no halogenation. On the other hand, the inactivation of barley α-amylase involves halogenation of tyrosine. The oxidative capabilities of these halogenating enzymes must be considered in any reaction system involving a functional activity. Unfortunately, oxidative reactions are much more difficult to observe because incorporation of radioactive substrates cannot be used to follow the reaction. Nevertheless, this study does indicate the usefulness of the peroxidative enzymes in uncovering useful and interesting information concerning structure-function relationships of other enzymes. In particular this is the first recognition of specific amino acid residues involved with the catalytic properties of these carbohydrate-hydrolyzing enzymes. At this moment the mechanism of catalysis is still elusive.

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