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AMINO ACID RESIDUES INVOLVED IN THE ACTION OF ENDO-(1→3)-β-D-GLUCANASE II FROM *Flavobacterium dormitator* var. *glucanolyticae* FA-5

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ABSTRACT

The pH-dependence of the kinetic parameters for the hydrolysis of yeast glucan with endo-(1→3)-β-D-glucanase II from *Flav. dormitator* var. *glucanolyticae* FA-5 suggests that two residues (histidyl and carboxyl) are involved in the enzyme action. Chemical modification of the enzyme has been studied in order to identify the kinds and the number of amino acid residues involved in enzyme action. Photo-oxidation and carbethoxylation of the enzyme indicated that the decomposition of a histidine residue is responsible for the loss of activity. Modification of the enzyme with Woodward's reagent K indicated that ~12 carboxyl residues in the enzyme are involved in the catalytic and/or substrate binding-site.

INTRODUCTION

Flavobacterium dormitator var. *glucanolyticae* FA-5 produces five endo-(1→3)-β-D-glucanases[1]. The purification and some physicochemical and enzymic properties of the enzymes II* and IV have been reported[1-3]. The large amounts of purified enzyme II available have allowed a study of the nature of the active site.

There is little information on the active site of endo-(1→3)-β-D-glucanase[4-7]. Identification of the residues involved in the enzyme action was difficult, because of the small amounts of enzyme available for study. Carboxyl and histidine residues are involved[5] in the active site of endo-(1→3)-β-D-glucanase IV, and histidine and the tryptophan side-chain are involved in the catalytic site[8] of *Basidiomycetes* exo-(1→3)-β-D-glucanase.

We now report on the chemical modification of amino acid residues of endo-(1→3)-β-D-glucanase II [E.C. 3.2.1.6, endo-1,3(4)-β-D-glucanase] and discuss the results in terms of enzyme structure and function.

MATERIALS AND METHODS

Enzyme. — Endo-(1→3)-β-D-glucanase II was prepared[2] from *Flavobacterium*

*The enzymes are denoted as B and I–IV, based on the order of elution from SP-Sephadex C-50 with 0.01M potassium phosphate buffer (pH 5.0).

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dormitator var. *glucanolyticae* FA-5; the $E^{1\%}_{1\,cm,\,280\,nm}$ value of 22.7 was used for determination of the enzyme concentration.

Reagents. — *N*-Ethyl-5-phenylisoxazolium-3′-sulfonate (Woodward's reagent K) (Fluka) was recrystallised three times from M HCl acetone. Mercaptoethanesulfonic acid and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) were commercial materials. Tetranitromethane was washed three times with an equal volume of water and distilled under reduced pressure. All other chemicals were special reagent grade and were recrystallised or distilled as appropriate.

Enzyme assay. — Enzyme activity was measured by using laminarin or yeast glucan as substrate[2,3].

Modification of the enzyme. — (*a*) Methionine residues were oxidised with chloramine T and *N*-chlorosuccinimide essentially by the procedure of Shechter *et al.*[10].

(*b*) Arginine residues were modified by treatment of the enzyme with 1,2-cyclohexanedione essentially as described by Patthy and Smith[11], except that the pH of buffer was lowered to 7.5 in order to stabilise the enzyme.

(*c*) Histidine residues were modified by photo-oxidation[12] of the enzyme (3 mg/mL) in the presence of 5×10^{-4}% of Rose Bengal.

(*d*) Carboxyl residues were modified by treatment[13] of the enzyme with EDC. Glycinamide was used as nucleophilic acceptor of the activated carboxyl groups.

(*e*) The carboxyl groups were modified by treatment[14] with Woodward's reagent K, and the reaction was quenched by the addition of 0.1 vol. of M sodium acetate (pH 6.0). The solution was dialysed against water and then hydrolysed in a sealed tube. The degree of modification was measured by the release of ethylamine (assayed using a Hitachi 034 amino acid autoanalyser) on acid hydrolysis.

(*f*) Tyrosine residues were modified by nitration of the enzyme in 0.01M Tris · HCl buffer (pH 8.0) at room temperature. 0.84M Tetranitromethane in 95% ethanol (10 μL) was added to 1 mL of a solution containing ~3 mg of the enzyme and 0.01M Tris · HCl buffer (pH 8.0).

For the measurement of the degree of modification and enzyme activity, aliquots were eluted from a column (0.9 × 15 cm) of Sephadex G-10 with 0.01M sodium acetate buffer (pH 6.0) to remove tetranitromethane. Nitrotyrosine residues of the enzyme were measured spectrophotometrically[15] and by amino acid analysis[16]. Reduction of nitrotyrosine residues of the enzyme was performed under the conditions described by Sokolovsky *et al.*[16].

(*g*) The enzyme was titrated[4] for mercapto groups and disulfide groups, and the number of mercapto groups reacted was based on a molar absorbance of 13,600 for the reduction product of 5,5′-dithiobis(2-nitrobenzoic acid)[17].

(*h*) Tryptophan residues and amino groups of the enzyme were modified under conditions similar to those reported by Robinson[18] and Klapper and Klotz[19], respectively, using 2-hydroxy-5-nitrobenzyl bromide and succinic anhydride.

Amino acid analysis. — Samples for amino acid analyses were precipitated with aqueous 5% trichloroacetic acid. The precipitate was washed with acetone and ether,

dried under nitrogen, and hydrolysed in sealed, evacuated tubes with 5.7M HCl or 3M mercaptoethanesulfonic acid for 24 h at 110°. Amino acids were analysed by using a Hitachi 034 amino acid autoanalyser. Methionine sulfoxide in the presence of methionine was determined[10,20] after treatment with cyanogen bromide in aqueous 80% formic acid.

RESULTS

pH-Dependence of kinetic parameters. — Fig. 1 shows a plot of the kinetic parameters against pH for the hydrolysis of laminarin. The plot of $\log(V_{max}/K_m)$ against pH gave two pK_e values (5.0 and 7.2). The pK_{e2} of the enzyme was not apparent in the plot of pK_m against pH. The pK of a group in the enzyme which may affect the velocity and be reflected in the plot of log V against pH will be cancelled out of the pK_m plot if its ionisation is not affected by binding with the substrate, as with glucoamylase[21].

Standard enthalphy changes for these two groups of the enzyme, as determined by the van't Hoff analysis, were 0 kcal/mol for the group having pK_{e1} 5.0 and +8 kcal/mol for the group having pK_{e2} 7.2, suggesting these groups to be a carboxyl group and histidine residue, respectively (Fig. 2).

These observations make it likely that carboxyl groups and histidine residue are parts of the catalytic mechanism.

Amino acid analysis. — Table I shows the amino acid composition of the enzyme.

Modification of methionine residues. — Table II shows that the addition of chloramine T to the enzyme at pH 8.0 did not cause loss of activity even with a large excess of reagent (molar ratio 2,500:1) and a longer incubation time (60 min)

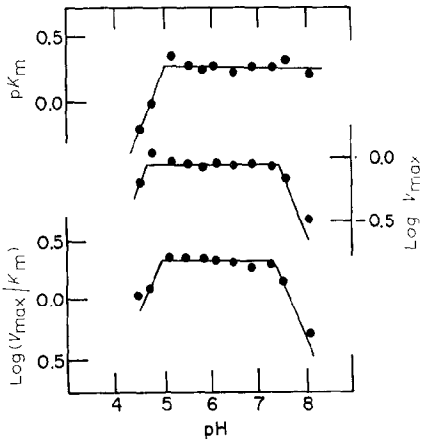

Fig. 1. Effect of pH on kinetic parameters.

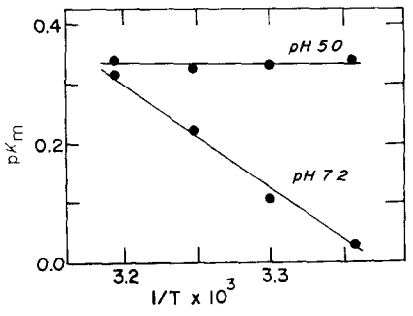

Fig. 2. Effect of temperature on pK_m.

TABLE I

AMINO ACID COMPOSITION OF ENDO-(1→3)-β-D-GLUCANASE II

Amino acid	*Time of hydrolysis (h)*			*Number of residues per 23,000 mol. wt.*[a]
	20	*40*	*60*	
	Amino acid (μmol/4.78 nmol of enzyme)			
Asp	0.116	0.108	0.118	24
Thr	0.088	0.081	0.078	18
Ser	0.068	0.057	0.053	15
Glu	0.101	0.095	0.103	21
Pro	0.071	0.065	0.072	15
Gly	0.132	0.124	0.128	28
Ala	0.112	0.100	0.110	23
Cys/2	(0.006)	(0.004)	(0.007)[b]	2
Val	0.052	0.057	0.059	12
Met	(0.009)	(0.008)	(0.009)[b]	2
Ile	0.025	0.026	0.029	6
Leu	0.050	0.047	0.052	11
Tyr	0.030	0.028	0.029	6
Phe	0.033	0.031	0.034	7
Lys	0.028	0.028	0.029	6
His	0.024	0.023	0.025	5
Arg	0.059	0.055	0.060	12
Trp	(0.024)[c]		(0.023)[d]	5

[a]Approximated to the nearest integer. [b]From performic acid-oxidised sample; determined as cysteic acid and methionine sulfone, respectively[9]. [c]Mercaptoethanesulfonic acid hydrolysis. [d]Spectrophotometric analysis.

TABLE II

MODIFICATION OF METHIONINE RESIDUES OF ENDO-(1→3)-β-D-GLUCANASE II WITH INCREASING QUANTITIES OF CHLORAMINE T[a]

Chloramine T/enzyme (Molar ratio)	*Methionine sulfoxide*	*Relative activity*
	Number of residues per 23,000 mol. wt.	
0	0	100
5	0.0–0.05	99–102
20	0.7–0.8	99–100
100	2.2–2.3	95–96
200	1.7–2.1	93–96
2,500	1.9–2.2	90–92
2,500[b]	1.7–2.1	93–95

[a]The enzyme (1.7 mg) was dissolved in 1.0 mL of 0.1M Tris·HCl (pH 8.0), and aqueous chloramine T was added. After 60 min, the protein was applied to Bio-Gel P-2, to remove chloramine T, and analysed for methionine sulfoxide content and enzyme activity. [b]The enzyme was modified in the presence of laminarin (5 mg/mL).

in the presence, or the absence, of laminarin as substrate, but there was rapid oxidation of 1.7–2.1 methionine residues (assayed as methionine sulfoxide).

The oxidation of <0.9 methionine residue of the enzyme with *N*-chlorosuccinimide was observed with 1,000 equiv. of *N*-chlorosuccinimide, but there was no loss of the enzyme activity.

Modification of arginine residues. — The enzyme did not lose its activity on treatment with 1,2-cyclohexanedione, but, after 1, 2, and 20 h, ~7 arginine residues had been modified. Arginine residues (11.1–12.4 residues/mol of enzyme) could be recovered from the modified enzyme by incubation at 37° in hydroxylamine buffer (pH 7.0) for 10 h, in good agreement with the recovery obtained by hydrolysis of the native enzyme with HCl.

Modification of histidine residues. — The photo-oxidation of the enzyme in the presence of Rose Bengal resulted in a loss of activity which followed pseudo-first-order kinetics (Fig. 3A). Laminarin protected against the photo-oxidation, but starch, glycogen, yeast mannan, or xylan had no effect. The rate of photo-oxidation was pH-dependent and the plots of the pseudo-first-order rate constant (k, min^{-1}) against pH fell close to the titration curves of histidine (Fig. 3B). Photo-oxidation of the enzyme in the absence of Rose Bengal had no effect on the activity.

By amino acid analyses or by titration with diazonium-1*H*-tetrazole, which has been used to quantify histidine and tyrosine[22], histidine was shown to be the only amino acid susceptible to photo-oxidation, and a loss of 1 residue/mol of the enzyme occurred. Fig. 4 shows that the decrease in enzyme activity was directly related to the amount of histidine residue modified. Amino acid analyses of the photo-oxidised enzyme revealed the destruction of 0.79 histidine residue/mol of enzyme with 90% loss of activity.

No loss of tryptophan was detected after photo-oxidation of the enzyme.

Methionine sulfoxide, which might be formed during the photo-oxidation, is reconverted into methionine during acid hydrolysis. Consequently, a sample of the

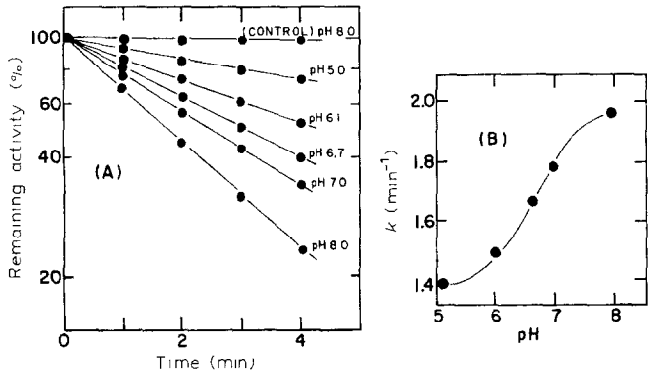

Fig. 3. Effect of pH on the rates of photo-oxidation of endo-(1→3)-β-D-glucanase II: (*A*) loss of enzyme activity at pH 5, 6.1, 6.7, 7, and 8; (*B*) effect of pH on the pseudo-first-order rate constant (k, min^{-1}) for photo-oxidation.

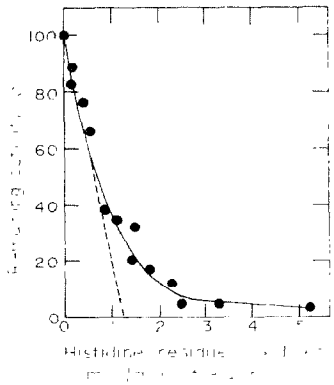

Fig. 4. Effect of photo-oxidation on the enzyme activity and the histidine content of endo-(1→3)-β-D-glucanase II. The enzyme was treated with 5 10^{-4}% Rose Bengal and analysed as described under Materials and Methods

photo-oxidised enzyme was hydrolysed with alkali. Amino acid analysis then revealed no methionine sulfoxide and no change in the methionine content of the protein.

Modification of carboxyl groups. - - Treatment of the enzyme with *N*-ethyl-5-phenylisoxazolium-3'-sulfonate (Woodward's reagent K) resulted in considerable precipitation. However, the rate of inactivation obeyed pseudo-first-order kinetics for the initial 3 min. No loss of enzyme activity was detected in control experiments carried out in the absence of Woodward's reagent K (Fig. 5). Fig. 5 shows also the

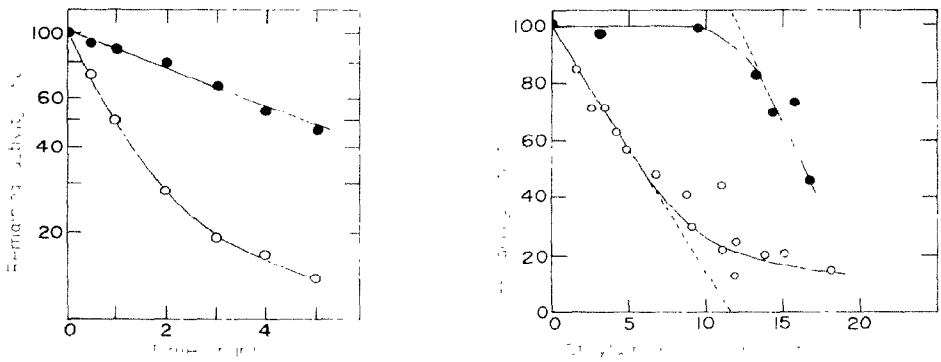

Fig. 5. Inhibition of endo-(1→3)-β-D-glucanase II with Woodward's reagent K. Reactions were carried out at pH 6 in 10 mM sodium phosphate and 2.2 mg of protein/mL at 5 . At intervals, 10-μL aliquots were withdrawn for enzyme assay. For substrate protection, mixtures contained 0.5% of laminarin: —●—, with laminarin; - - -, without laminarin

Fig. 6. Effect of the degree of reaction with Woodward's reagent K on enzyme activity. The enzyme (6.6 mg) was treated with a 500-fold excess of Woodward's reagent K as described in Fig. 5. Ethylamine determinations were performed after stopping the reaction with 98% formic acid: —●—, with laminarin; . without laminarin

substrate protection of the enzyme with laminarin. Other (1→3)-β-D-glucans, such as pachyman and yeast glucan, also protected the enzyme from Woodward's reagent K, but glycogen, starch, and pullulan had no effect. Fig. 6 shows that the essential carboxyl residues for the hydrolysis of (1→3)-β-D-glucan are 12 reactive groups out of a total 45 carboxyl residues (including amide groups). Amino acid analyses revealed that no other residues were modified. These results suggest that the carboxyl group is at the active site and/or substrate binding-site, or is involved in maintenance of structural integrity.

Also, the enzyme was rapidly inactivated by 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC).

Modification of tyrosine residues. — No loss of activity could be detected after treatment of the enzyme at pH 8.0 with a large excess of tetranitromethane (molar ratio $10^5:1$) and a long incubation time (5 h), which led to a nitration of 2.4–3.0 tyrosine residues as determined by spectrophotometric and amino acid analyses, indicating that 3 tyrosine residues of the enzyme were exposed and the other 3 residues were buried in the interior of the enzyme protein (Table III). The maximum nitration corresponding to 6.1 nitrotyrosine residues/mol of enzyme was achieved when the nitration was performed in 5M guanidinium hydrochloride.

Modification of cysteine residues. — Modification of cysteine residues with 5,5′-dithiobis(2-nitrobenzoic acid) did not cause any loss of enzyme activity. The number of total cysteine residues analysed was 1.6–1.8 in the reduced protein, in good agreement with the numbers obtained by carboxymethylation.

Modification of tryptophan residues and amino groups. — The activity of the enzyme decreased by ~20%, after modification of the enzyme with >80 mol of 2-hydroxy-5-nitrobenzyl bromide, and with succinic anhydride.

DISCUSSION

Enzyme kinetics and chemical modification have been used to identify amino

TABLE III

NITRATION OF ENDO-(1→3)-β-D-GLUCANASE II WITH TETRANITROMETHANE

Preparation	*Nitrotyrosine*[a] *(mol/mol of enzyme)*		*Relative enzyme activity*
	I	*II*	
Enzyme nitrated in the absence of Guan[b]	2.4–3.0	3.1–3.2	80
Enzyme nitrated in the presence of Guan	5.8–6.1	6.0–6.1	0
Native enzyme	0.0	0.0	100

[a]I, Spectrophotometric analysis at 428 nm; II, amino acid analysis. [b]Guanidinium hydrochloride.

acid residues involved in the enzyme action of endo-(1→3)-β-D-glucanase II from *Flav. dormitator* var. *glucanolyticae* FA-5, and have been compared with similar attempts to modify amino acid residues in *Basidiomycetes* exo-(1→3)-β-D-glucanase[8].

The first-order loss of enzyme activity during photo-oxidation is evidence that the decomposition of a single type of functional group is occurring. Although tyrosine, tryptophan, methionine, cysteine, and histidine have been reported to be affected by photo-oxidation, attempts were made to rule out tyrosine, tryptophan, methionine, and cysteine by the use of selective reagents

Nitration of the enzyme with tetranitromethane indicated that 3 out of 6 tyrosine residues are reactive. The nitrated enzyme retained 80% of the activity of the native enzyme. These reactive tyrosine residues are located outside the active site and the sub-site cleft. The small decrease in activity may be due to small conformational changes caused by the modification.

Although tetranitromethane also reacts with cysteine and methionine residues, the loss of the enzyme activity was not due to modification of these residues, since treatment of the enzyme with chloramine T, *N*-chlorosuccinimide, and 2-hydroxy-5-nitrobenzyl bromide had no effect on activity.

Oxidation of proteins with *N*-chlorosuccinimide at pH 7.0–8.5 can distinguish[10] between exposed, partially exposed, and buried methionine residues. A single methionine residue was oxidised with 1,000 equiv. of *N*-chlorosuccinimide, indicating that this residue was completely exposed and that the other 3 methionine residues were buried within the enzyme molecule.

Thus, only a small part, if any, of the loss of enzyme activity on photo-oxidation can be attributed to the oxidation of methionine, tyrosine, cysteine, and tryptophan. The loss of activity which accompanied the destruction of one histidine residue per enzyme molecule indicates that this residue is essential for activity.

The 7 arginine residues of the enzyme reacted with 1,2-cyclohexanedione and there was no loss of activity. Thus, the arginine residues have no role in the activity of the enzyme.

Modification of the carboxyl group of the enzyme with Woodward's reagent K or EDC, and the protection with laminarin, pachyman, and yeast glucan may be compared with the results of similar experiments conducted on *Basidiomycetes* exo-(1→3)-β-D-glucanase[8]. Carboxyl groups are not involved in catalysis effected by the *Basidiomycetes* enzyme, whereas the carboxyl groups of *Flav.* endo-(1→3)-β-D-glucanase II and IV are protected by the binding of such substrates as laminarin, pachyman, or yeast glucan, and appear to play a role in catalysis, substrate binding, and/or in maintenance of structural integrity. The present finding makes it likely that carboxyl groups in endo-(1→3)-β-D-glucanase II most effectively protected from modification by binding of substrate are involved in the substrate binding-site and probably promote hydrogen bonding. Endo-(1→3)-β-D-glucanase II has 12 carboxyl groups essential for the catalysis.

Thus, it is concluded that the carboxyl groups and the protonated imidazole ring of histidine are essential for the action of *Flav.* endo-(1→3)-β-D-glucanase II.

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