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PURIFICATION AND PROPERTIES OF AN EXO-(1→3)-β-D-GLUCANASE FROM THE CULTURE FILTRATE OF *Mucor hiemalis*

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ABSTRACT

An exo-(1→3)-β-D-glucanase (an-1), isolated from the culture filtrate of *Mucor hiemalis N.O.*, has been purified by precipitation with ammonium sulfate and successive chromatography on Sephadex G-100, DEAE-Sephadex A-25, and Laminarin-AH-Sepharose 4B. The purified enzyme had an apparent molecular weight of 75,000 in SDS-polyacrylamide gel electrophoresis. The optimum pH value was 5.5 and the optimum temperature was 40°. The enzyme was stable in the pH range of 4.5–8.0 and at <45°, and had an exo-type action pattern yielding glucose as the only product of low mol. wt. The enzyme was deactivated by 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodi-imide methotoluene-*p*-sulfonate and *N*-bromosuccinimide, which implicated carboxyl group(s) and tryptophan residue(s) in the catalytic function.

INTRODUCTION

(1→3)-β-D-Glucan has been used clinically as an immunomodulating anti-cancer drug. Studies of the primary structure and conformation of this glucan could lead to an understanding of the mechanism of action. (1→3)-β-D-Glucanases [(1→3)-β-D-glucanhydrolases] play a major role in the production of yeast protoplasts, and the purification of these enzymes and the characterisation of their active sites are of interest because of their action on yeast cell walls and their use in the structural analysis of the glucans. (1→3)-β-D-Glucanases are ubiquitous and they have been studied extensively[1–11].

The amino acid residues involved in the catalytic activity of glucan-hydrolysing enzymes, such as amylase and lysozyme, have been identified[12–13], but little is known about the catalytic sites of (1→3)-β-D-glucanases. We now report on the purification, properties, and active sites of exo-(1→3)-β-D-glucanase from *Mucor hiemalis* induced by the (1→3)-β-D-glucan, laminarin.

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EXPERIMENTAL

Micro-organism and enzymes. — *Mucor hiemalis N.O.* was used for the production of enzymes by submerged culture in a medium containing 0.2% of laminarin, 0.2% of $(NH_4)_2SO_4$, 0.2% of KH_2PO_4, 0.1% of $MgSO_4 \cdot 7\ H_2O$, and 0.01% of yeast extract[14] in a 10-L jar-fermentor (Microferm Laboratory Fermentor MF-114). The culture broth was filtered through a 25G-2 glass filter, and the filtrate was used as the enzyme source.

Enzyme assay. — The reaction mixture containing 0.1% of laminarin, 0.1M acetate buffer (pH 5.5), and enzyme solution was kept at 40° for 60 min. The reducing sugar produced was determined by literature methods[15–17]; 1 unit (U) of enzyme activity was defined as the amount of enzyme that produced 1 μmol of reducing sugar as glucose in 60 min under these conditions.

Protein assay. — Protein concentrations were determined by the method of Warburg and Christian[18], and column fractions were assayed for protein on the basis of the extinction at 280 nm.

Substrates. — Laminarin (from *Eisenia araborea)* was a commercial product, and grifolan NMF-5N was purified[19] from *Grifola frondosa*. Islandican was prepared by the method of Nakamura *et al.*[20], and oligocurdlan (d.p. 20) by mild acid hydrolysis of curdlan.

Affinity chromatography. — Laminarin-AH-Sepharose 4B, used for substrate affinity chromatography, was prepared by the method of Usui *et al.*[21].

General properties of the enzymes. — (*a*) *Effect of pH.* Acetate, Tris-HCl, and carbonate ($NaHCO_3$–Na_2CO_3) buffers in the pH ranges of 3.5–6.5, 7.0–8.0, and 9.0–11.0, respectively, were used. The enzyme was treated with each buffer (50 mM) for 18 h at 18° in order to determine its stability. The optimum pH was determined by use of each buffer (0.1M) under the standard conditions.

(*b*) *Effect of temperature.* The activity remaining was assayed under the standard conditions after the enzyme had been kept in 0.1M acetate buffer (pH 5.5) for 10 min at temperatures in the range 30–70°.

(*c*) *Effect of metal ions.* The enzyme activity was examined in the presence of the following salts in the range 10^{-2}–10^{-4}M: $MgCl_2 \cdot 7\ H_2O$, $CaCl_2 \cdot 2\ H_2O$, $FeCl_3 \cdot 6\ H_2O$, $NiSO_4 \cdot 7\ H_2O$, $HgCl_2$, $ZnSO_4 \cdot 7\ H_2O$, $CuCl_2 \cdot 2\ H_2O$, $SnCl_2 \cdot 2\ H_2O$, and $Pb(OAc)_2 \cdot 3\ H_2O$. The relative activity was expressed as a percentage of the enzyme activity in the absence of metal ions.

(*d*) *Substrate specificity.* The standard assay conditions were used together with the following substrates: 0.1% laminarin, grifolan NMF-5N, oligocurdlan, islandican, pullulan, dextran, and methyl α- and β-D-glucopyranoside.

(*e*) *Molecular weight of the enzyme.* SDS polyacrylamide gel electrophoresis was employed[22] with bovine serum albumin (mol. wt. 68,000), aldolase (40,000), and trypsin-inhibitor (21,500) as standards.

(*f*) K_m *value.* A Hanes–Woolf plot was used as follows. The value of the substrate concentration was divided by the reducing power after incubation for 60 min (as the ordinate) and the concentration of substrate (as the abscissa).

(*g*) *Mode of action of the enzyme.* A solution of laminarin (5.5 mg) in 0.1M acetate buffer (pH 5.6, 0.5 mL) was preincubated for 10 min at 40°. The enzyme solution (0.7 U/mL) was then added and incubation was continued for 3 or 46 h at 40°. After inactivation of the enzyme by heating for 3 min at 100°, the incubation mixture was concentrated and applied to a column (1.7 × 143 cm) of Bio-Gel P-2 with water. Fractions (2.6 mL) were assayed by the phenol–sulfuric acid method[23].

Chemical modification. — (*a*) *By succinic anhydride*[24]. A mixture of 0.1M borate buffer (pH 7.5) and enzyme (31.4 U/mg) was made 25mM in succinic anhydride and stored at room temperature for 60 min. The reaction was stopped by the addition of distilled water.

(*b*) *By 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodi-imide methotoluene-p-sulfonate (CMC).* A procedure similar to that of Irie *et al.*[25,26] was used. To a solution of the enzyme (76.5 U/mg) in 0.1M 3-(*N*-morpholino)propanesulfonic acid buffer was added CMC to 10mM at room temperature, and the solution was stored for 30 min. Aliquots, withdrawn at intervals, were each added to acetate buffer (0.1 mL, pH 5.0), giving a final concentration of 0.2M in order to stop the reaction.

(*c*) *By 1,2-cyclohexanedione*[27]. A mixture of 0.2M borate buffer (pH 7.5), enzyme (76.5 U/mg), and 10mM 1,2-cyclohexanedione was stored at 37° for 60 min.

(*d*) *By sodium tetrathionate*[28,29]. A mixture of enzyme (76.5 U/mg), 20mM acetate buffer (pH 6.5), and 10mM 2-mercaptoethanol was kept at 37° for 30 min. Sodium tetrathionate was then added to 0.168mM and the solution was kept at room temperature for 5 min.

(*e*) *By diethyl pyrocarbonate*[30]. A mixture of 0.1M Tris-HCl (pH 7.0), enzyme (31.4 U/mg), and 2mM diethyl pyrocarbonate was kept at room temperature for 30 min. The diethyl pyrocarbonate was added in the buffer containing 4% of acetonitrile.

(*f*) *By* N-*bromosuccinimide*[31,32]. The imide was added (to 20mM) to the enzyme solution (76.5 U/mg) in 0.1M acetate buffer (pH 5.0), and the mixture was kept for 30 min at room temperature.

RESULTS AND DISCUSSION

Production of (1→3)-β-D-glucanase from Mucor hiemalis N.O. — The organism was cultured at pH 5.35 (see Experimental) in a 10-L jar-fermentor at 25°, with an air supply of 9 L/min. Fig. 1 shows the time course of cultivation. The (1→3)-β-D-glucanase activity in the culture reached a maximum (0.64 U/mL) at 30 h.

After 30 h, the mycelium was removed (25G-2 glass filter) and the culture filtrate (~9 L, pH 3.7) was concentrated (to 500 mL) using a Hollow Fiber (H1P10-20, Amicon). Insoluble material was removed by centrifugation at 15,000 r.p.m. for 15 min.

The enzyme was precipitated by saturation of the supernatant solution with $(NH_4)_2SO_4$ and dissolved in 0.1M phosphate buffer (pH 7.2). As shown in Table I,

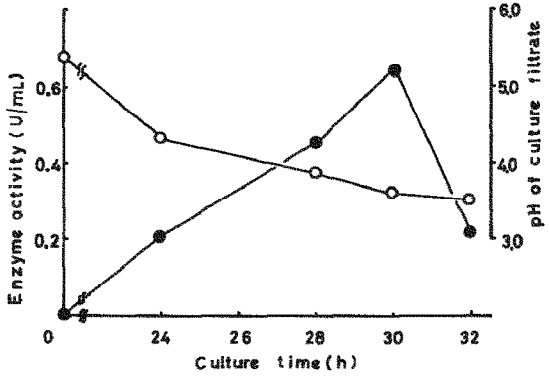

Fig. 1. Time course of the production of (1→3)-β-D-glucanase by *Mucor hiemalis N.O.*: —○—, pH; —●—, enzyme activity.

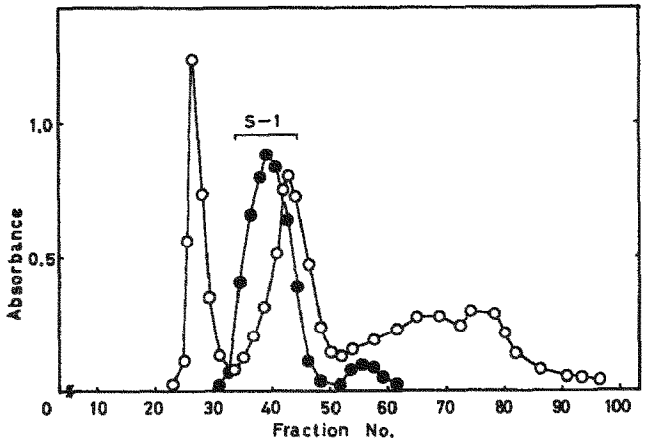

Fig. 2. Gel filtration of the crude (1→3)-β-D-glucanase on a column (1.7 × 85 cm) of Sephadex G-100, using 0.1M phosphate buffer (pH 7.2) (2.6-mL fractions): —○—, absorbance at 280 nm; —●—, enzyme activity.

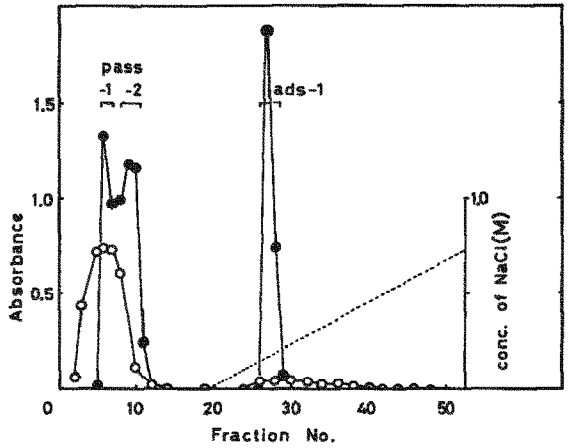

Fig. 3. Ion-exchange chromatography of S-1 on a column (1.9 × 5.0 cm) of DEAE-Sephadex A-25 (2.7 mL fractions) :—○—, absorbance at 280 nm; —●—, (1→3)-β-D-glucanase activity; ------, NaCl concentration.

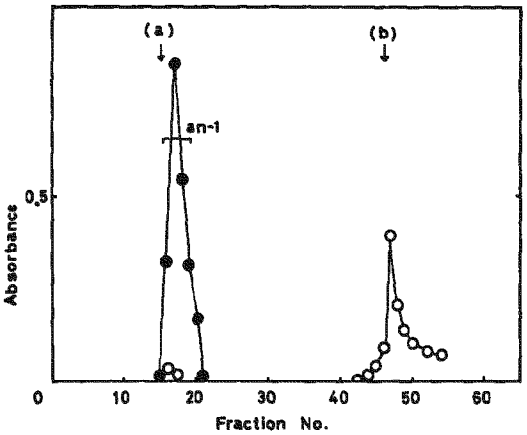

Fig. 4. Substrate-affinity chromatography of ads-1 on a column (1.4 × 2.3 cm) of laminarin-AH-Sepharose 4B: elution with 50mM acetate buffer (pH 5.6) containing (a) M NaCl, (b) M NaCl and 0.1% of laminarin (3.1-mL fractions): —○—, absorbance at 280 nm; —●—, (1→3)-β-D-glucanase activity.

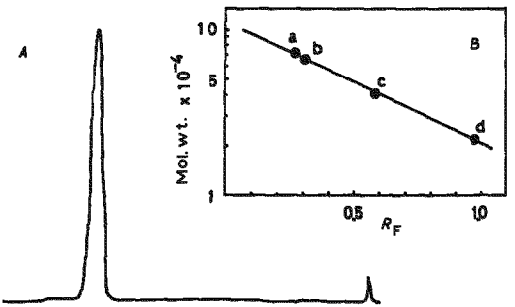

Fig. 5. *A*, A densitometric scan of (1→3)-β-D-glucanase; *B*, mobilities in SDS polyacrylamide gel electrophoresis[22]: *a*, (1→3)-β-D-glucanase; *b*, bovine serum albumin (mol. wt. 68,000); *c*, aldolase (40,000); and *d*, trypsin-inhibitor (21,500).

TABLE I

PURIFICATION OF THE EXO-(1→3)-β-D-GLUCANASE

	Protein (mg)	*Activity (U)*	*Specific activity (U/mg)*	*Yield (%)*	*Purification (fold)*
Culture filtrate	2096.9	1071.6	0.5	100.0	1.0
$(NH_4)_2SO_4$ fraction	17.4	190.9	11.0	17.8	21.5
Sephadex G-100, S-1	4.6	87.3	18.9	8.2	37.1
DEAE-Sephadex					
pass-1	0.1	2.5	17.5	0.3	34.2
pass-2	0.1	1.7	19.3	0.2	37.9
ads-1	0.2	4.9	24.8	0.5	48.6
Laminarin-AH-Sepharose 4B					
an-1	0.03	3.8	111.1	0.4	217.9

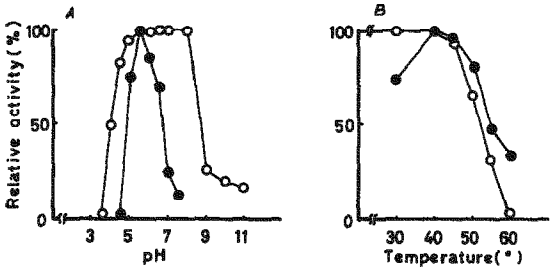

Fig. 6. *A*, pH stability (—○—) and optimum pH (—●—) of the (1→3)-β-D-glucanase; *B*, thermostability (—○—) and optimum temperature (—●—).

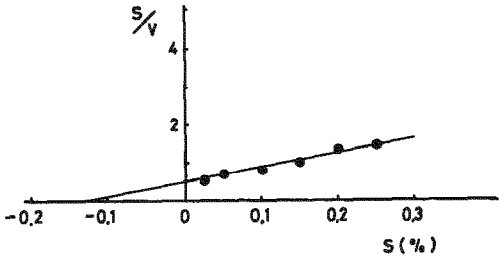

Fig. 7. Hanes–Woolf plot of the initial velocity of the (1→3)-β-D-glucanase.

>50% of the original enzyme activity was lost. The solution was eluted from a column (1.7 × 85 cm) of Sephadex G-100 with 0.1M phosphate buffer (pH 7.2) (Fig. 2). The fractions containing (1→3)-β-D-glucanase were combined, concentrated by ultrafiltration (PM-10, Amicon), and applied to a column (1.9 × 5.0 cm) of DEAE-Sephadex A-25, using 10mM acetate buffer (pH 6.0). The column was eluted with a linear gradient (0→M NaCl). As shown in Fig. 3, three forms of the enzyme were obtained (pass-1, pass-2, and ads-1). The fraction ads-1 was purified

TABLE II

EFFECTS OF METAL IONS ON THE ACTIVITY OF AN-1

	Relative activity (%)		
	(1×10^{-2}M)	*(1×10^{-3}M)*	*(1×10^{-4}M)*
$MgCl_2$	98.9	119.9	90.3
$CaCl_2$	92.7	101.1	97.3
$FeCl_3$	8.6	53.4	85.2
$NiSO_4$	0	63.6	81.7
$HgCl_2$	0	14.8	64.7
$ZnSO_4$	2.7	28.3	49.6
$CuCl_2$	3.3	16.3	29.7
$SnCl_2$	0	0	37.2
$Pb(OAc)_2$	0	42.0	86.3
None	100	—	—

TABLE III

SUBSTRATE SPECIFICITY OF AN-1 ON D-GLUCANS AND METHYL D-GLUCOPYRANOSIDES

Substrate	*Relative activity (%)*	*Substrate*	*Relative activity (%)*
Laminarin [(1→3,1→6)-β]	100	Pullulan [(1→4,1→6)-α]	0
Grifolan NMF-5N [(1→3,1→6)-β]	8	Dextran T-10 [(1→6)-α]	0
Curdlan (d.p. 20) [(1→3)-β]	5	Methyl α-D-glucopyranoside	0
Islandican [(1→6)-β]	0	Methyl β-D-glucopyranoside	0

further since it showed most of the activity. It was loaded on to a column (1.4 × 2.3 cm) of Laminarin-AH-Sepharose 4B, equilibrated with 50mM acetate buffer (pH 5.6) and eluted with this buffer containing M NaCl (Fig. 4). The resulting enzyme preparation (an-1) was homogeneous by SDS-PAGE (Fig. 5) and its mol. wt., as determined by the method of Laemmli[22], was ~75,000.

General properties of the enzyme. — As shown in Fig. 6, the enzyme was most active at pH 5.5 and was stable in the pH range of 4.5–8.0, showed maximum activity at 40°, and was stable at <45°. The activity was lost at 55°. As shown in Table II, the enzyme was not affected by Mg^{2+} and Ca^{2+} ions, but was inhibited moderately by Fe^{3+}, Ni^{2+}, Zn^{2+}, Pb^{2+}, and Hg^{2+} ions, and strongly inhibited by Cu^{2+} and Sn^{2+} ions. The action of the enzyme on various polysaccharides, as shown in Table III, was compared to that on laminarin (100%). Action of the enzyme on grifolan NMF-5N [an antitumor (1→3)-β-D-glucan] and oligocurdlan was ~8.1 and ~4.8%, respectively. Other substrates were not degraded by the enzyme. The K_m value of the enzyme, as obtained by a Hanes–Woolf plot, was 0.125% (Fig. 7).

Under physiological conditions, laminarin is thought to possess a random-coil conformation and curdlan a single- and multi-stranded helix structure. It is assumed

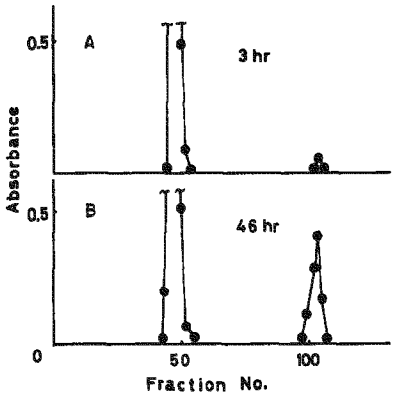

Fig. 8. Gel-filtration pattern of enzyme digests (*A*, 3 h; *B*, 46 h) of laminarin on a column (1.7 × 143 cm) of Bio-Gel P-2. Laminarin (5.5 mg) was incubated at 40° and pH 5.6 in the presence of the enzyme (0.7 U/mL), and the hydrolysate was eluted from the column with water (2.6-mL fractions). Laminarin (V_o) and glucose were eluted at fractions 47 and 104, respectively.

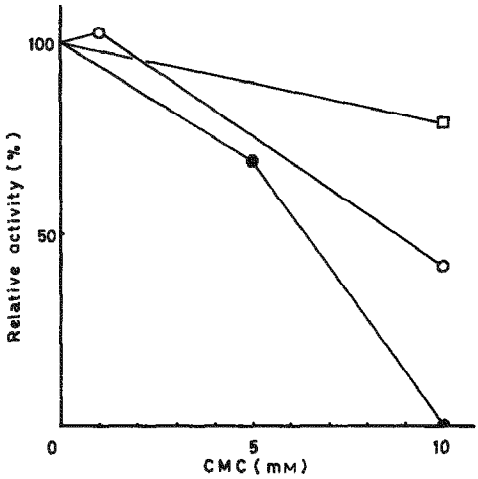

Fig. 9. Protective effect of laminarin (—●—, 0%; —○—, 0.1%; —□—, 1%) against the inactivation of (1→3)-β-D-glucanase treated with 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodi-imide methotoluene-*p*-sulfonate (CMC).

that the activity of the enzyme would affect not only the primary structure but also the conformation of the glucan chains. Laminarin was the substrate most readily hydrolysed by the enzyme, and Fig. 8 shows the elution patterns of the enzymic hydrolysate of laminarin from a column of Bio-Gel P-2. After incubation for 3 and 46 h, glucose was the only product of low mol. wt. (identified by t.l.c.). Therefore, the enzyme appears to be an exo-(1→3)-β-D-glucanase. Glucose was recovered in a yield of only ~10%, and the reaction would be incomplete because only a small quantity (0.7 U) of enzyme was added. Further quantitative work could not be done because of the limited yield of the enzyme.

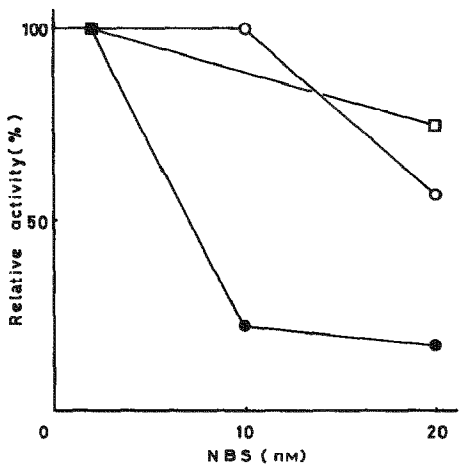

Fig. 10. Protective effect of laminarin (—●—, 0%; —○—, 0.1%; —□—, 1%) against the inactivation of (1→3)-β-D-glucanase treated with *N*-bromosuccinimide (NBS).

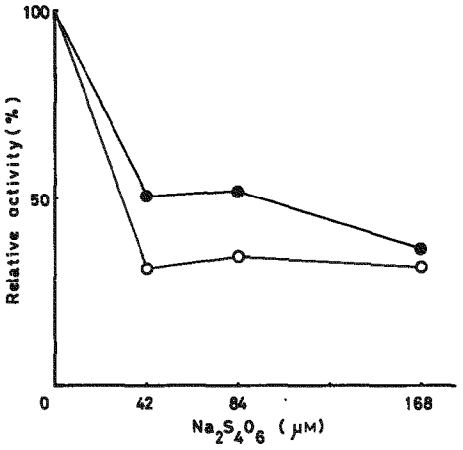

Fig. 11. Protective effect of laminarin (—●—, 0%; —○—, 0.1%) against the inactivation of (1→3)-β-D-glucanase treated with sodium tetrathionate.

Several reactions were used to investigate the active site of the enzyme (Table IV). Only 1-cyclohexyl-3-(2-morpholinyl-4-ethyl)carbodi-imide methotoluene-*p*-sulfonate (CMC), *N*-bromosuccinimide (NBS), and sodium tetrathionate completely inactivated the enzyme. In the presence of laminarin (0.1 and 1.0%), the enzyme was protected from inactivation by CMC in a dose-dependent manner (Fig. 9) and by NBS (activity remained at 60 and 75%, respectively) (Fig. 10). However, the inactivation of the enzyme with sodium tetrathionate was not inhibited in the presence of laminarin (Fig. 11). These results suggest that a carboxyl group(s) and a tryptophan residues(s) are present at or near the active site of the enzyme.

ACKNOWLEDGMENTS

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TABLE IV

EFFECTS OF CHEMICAL MODIFICATION ON THE ACTIVITY OF AN-1

Reagent	*Residue*	*Remaining activity (%)*
Succinic anhydride	$-NH_2$ (Lys)	95
CMC[a]	-COOH (Asp, Glu)	0
1,2-Cyclohexanedione	Arg	93
Sodium tetrathionate	Cys	31
Diethyl pyrocarbonate	His	106
N-Bromosuccinimide	Trp	18

[a]1-Cyclohexyl-3-(2-morpholinylethyl)carbodi-imide methotoluene-*p*-sulfonate.

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