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ESSENTIAL CARBOXYL AND TRYPTOPHAN RESIDUES IN JACK BEAN α-D-MANNOSIDASE

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Key Word Index—*Canavalia ensiformis*; Leguminosae; Jack Bean; modification; glycosidase; α-mannosidase.

Abstract—α-Mannosidase from *Canavalia ensiformis* was chemically modified using carboxyl group-specific carbodiimides and tryptophan specific *N*-bromosuccinimide. Data consistent with the presence of one essential carboxyl residue and 4 tryptophan residues was obtained. *N*-terminal sequencing studies indicated that the enzyme exists as a dimer with subunit M_r of 110,000. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

α-D-Mannosidase (EC 3.2.1.24) from Jack Bean (*Canavalia ensiformis*) has been partially characterized [1]. It is a metallo-enzyme, requiring zinc for catalytic activity [2] and is thought to exist as a tetramer of 2 polypeptides of M_r 66,000 and 44,000 [1]. It is a non-linkage-specific glycosidase and is able to cleave α-1,2′-, α-1,3′-, and α-1,6′-linked oligosaccharides [3].

The amino acid composition of the enzyme has been determined [4] and we have now identified the amino terminal sequence, although the entire sequence is still unknown. It was shown to have no cysteine residues, but a high proportion of acidic amino acids as is usual for enzymes from protein bodies. Most glycosidases characterized to date utilize a reaction mechanism similar to that of lysozyme [5]. This mechanism relies on the presence of two carboxyl-containing amino acids which act as general acid catalyst and nucleophile respectively [5].

Tyrosine (Tyr) and tryptophan (Trp), thought to play a major role in carbohydrate binding [6], represented 4.81 and 1.84 mol% respectively, which is similar to *Phaseolus vulgaris* α-mannosidase [7]. Here we present an investigation into the chemistry of the active site of Jack Bean α-mannosidase.

RESULTS AND DISCUSSION

The α-mannosidase from Jack Bean is thought to exist as a tetramer composed of subunits of M_r 44,000 (β-subunit) and 66,000 (α-subunit) [1]. Some reports, however, claim that the enzyme is a dimer of two subunits of M_r 110,000 [8]. This confusion results from the fact that the enzyme appears as two bands (44,000 and 66,000) on SDS PAGE only after boiling in the presence of a denaturant. On native PAGE or after heating at 37° the enzyme appears as a single band of M_r 110,000. It is unclear from the published literature whether the enzyme exists in subunit form or if the two bands seen on SDS PAGE result from a cleavage event on boiling in denaturant. In order to clarify the situation, the two apparent subunit bands from an SDS gel and the single band from a native gel were excised and partially sequenced. The *N*-terminal sequence from the apparent α-subunit (M_r 66,000) was Met Lys Tyr Asn Thr Gly Ala Gly Thr Val Pro Glu Lys Leu Asn. The apparent β-subunit (M_r 44,000) gave unreproducible data, with the following sequences being recorded: Ser X Thr Ile Asn Ile Gly; X Gln Glu Thr Ile Asn Ile Gly Pro Asp Leu Lys Met Ser Phe (where X is unknown). The *N*-terminal sequence of the single band from the native gel was identical with the *N*-terminal sequence from the α-subunit. No other *N*-termini could be detected in this band. These *N*-terminal sequencing studies suggest that the enzyme exists as a single subunit with a M_r of 110,000. This hypothesis is supported by the observation that the β-subunit gave unreproducible sequence data, consistent with a relatively non-specific cleavage event occurring upon boiling in SDS.

A preliminary survey of the effect on activity of several protein modification reagents was carried out (Table 1). The only reagents found to significantly decrease the activity of the enzyme were the carboxyl-specific reagents Woodwards Reagent K and the

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Table 1. Effect of protein modification reagents on enzyme activity

Modification reagent	Target amino acid residue	Enzyme concentration (u/ml)	Reagent concentration (mM)	pH	% residual activity
2-Hydroxy-5-nitrobenzyl-bromide (HNBB)	Tryptophan	0.08	10	4	87
N-Bromosuccinimide (NBS)	Tryptophan	0.08	10	7	0
		0.08	1	7	0
Diethyl-pyrocarbonate (DEPC)	Histidine	0.08	10	7	100
Woodwards reagent K (WWK)	Carboxyl amino acids	0.08	10	6	60
EDCI (1-ethyl-3,3-dimethyl aminopropylcarbodiimide methiodide)	Carboxyl amino acids	0.1	100	6	74
		0.1	300	6	46
EDAC (1-ethyl-3,3-dimethyl aminopropylcarbodiimide)	Carboxyl amino acids	4	300	6	50
		4	100	6	20
N-Acetylimidazole (NAI)	Tyrosine	0.1	60	6.5	95
Tetranitromethane (TNM)	Tyrosine	0.1	20	8	98

A stock of enzyme (4 mg/ml) was incubated with the appropriate modifier and buffer for 15 minutes. 0.1 μl sample was taken and assayed for α-mannosidase activity.

water-soluble carbodiimides 1-ethyl-3,3-dimethyl-aminopropylcarbodiimide methiodide (EDCI) and 1-ethyl-3,3-dimethylaminopropylcarbodiimide (EDAC); and the tryptophan-specific reagent *N*-bromosuccinimide (NBS). The inactivation of the enzyme by these reagents was investigated further.

Inactivation of the enzyme by the water-soluble carbodiimides, EDCI and EDAC, was investigated by following the percentage remaining activity as a function of time for several concentrations of EDCI (Fig. 1) and EDAC (Fig. 2). The loss of activity was found to be time and concentration dependent. EDAC gave greater inactivation than EDCI and this reagent was used for further study. The rate constants obtained from Fig. 2 were plotted as a function of EDAC concentration according to Levy *et al.* [9] (Fig. 3). The line on this plot has a gradient of 1.13 indi-

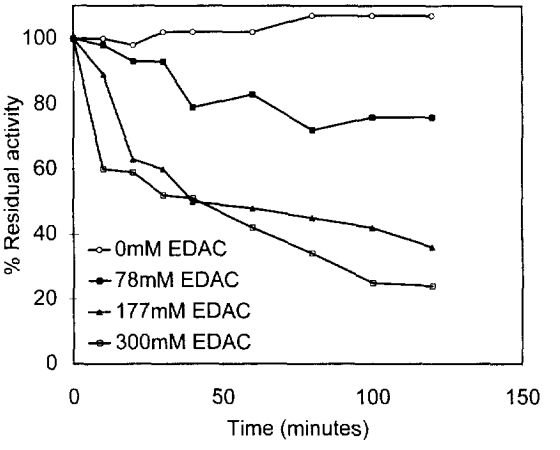

Fig. 2. Inactivation of α-mannosidase with EDAC. Various concentrations of EDAC were incubated with 2 ml of enzyme in 0.05 M MES-NaOH, pH 6. Enzyme activity was measured at time intervals.

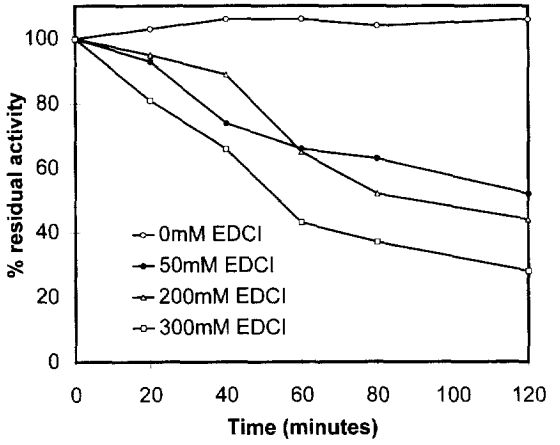

Fig. 1. Inactivation of α-mannosidase with EDCI. Various concentrations of EDCI were incubated with 2 ml of enzyme in 0.05 M MES-NaOH, pH 6. Enzyme activity was measured at time intervals.

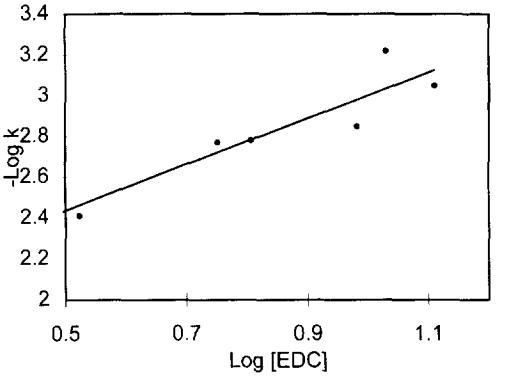

Fig. 3. Levy plot of apparent rate constants from Fig. 2 against EDAC concentration. The slope (calculated from a regression analysis) has a gradient of 1.13 suggesting a reaction between one critical carboxyl residue and one molecule of inactivating reagent.

cating that one molecule of EDAC reacted with one molecule enzyme, suggesting the existence of one carboxyl group that is essential for activity. In many reported studies of the inactivation of enzymes with carbodiimides, inactivation occurs when the reaction mixture contains an added nucleophile. Addition of 500 mM methylamine as a nucleophile had no effect on rate of reaction (data not shown). These results suggest that the carbodiimides are reacting with a catalytically essential carboxyl group. In order to prove that the modified residues are at the active site, it is necessary to demonstrate that the rate of inactivation of the enzyme can be decreased by incorporation of substrate into the reaction mixture. No protection from inactivation could be observed when yeast mannan, *p*-nitrophenyl-α-mannopyranoside, or methyl-α-mannopyranoside were used as substrates.

One possible explanation for the lack of protection of the carboxyl groups might be the high content of acidic groups in this protein. Conceivably, modification of a large number of carboxyl groups in the vicinity of the active site might result in steric hindrance of the approach of substrate to the active site. It is also conceivable that extensive modification of the carboxyl groups causes conformational changes in the protein, resulting in loss of activity.

The potential involvement of tryptophan residues was investigated further by titration with NBS. Figure 4 shows the titration behaviour of the enzyme with 0.3 mM NBS. The conversion of the indole ring of tryptophan to oxindole was followed by measurement of absorbance at 280 nm. Figure 4 shows a drop in A_{280} with time. As can be seen, the activity of the enzyme falls concomitantly with the fall in A_{280} resulting in almost complete loss of activity.

Tryptophan residues are essential for substrate binding in many glycosidases, including lysozyme [11], glucoamylase [12], cellulase [13] and xylanase [14].

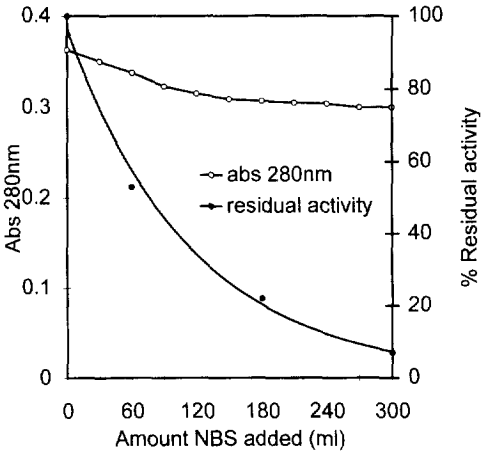

Fig. 4. Inactivation of α-mannosidase with NBS. Titration curve showing the decrease in A_{280} and activity when increasing amounts of 0.3 mM NBS were added to 0.2 ml enzyme (4 mg/ml) and 1.5 ml 25 mM sodium acetate buffer pH 4.8.

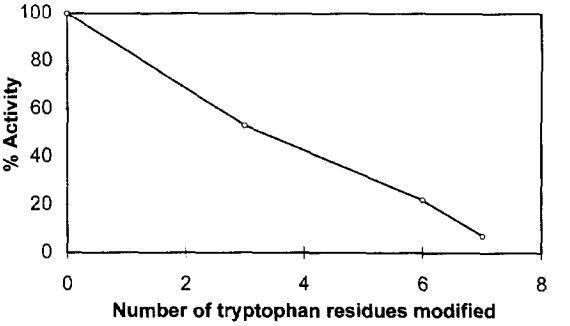

Fig. 5. Inactivation as a function of number of tryptophans modified. The number of residues oxidised by titration with 0.3 mM *N*-bromosuccinimide was calculated using the method of Spande and Witkop [10] with the M_r of the enzyme taken to be 220,000.

Low concentrations of *N*-bromosuccinimide were used in order to avoid an increase in A_{280} [15]. The number of modified tryptophan residues can only be calculated from a decrease in absorbance even though the activity may still be decreasing. Using 0.3 mM NBS a decrease to 7% (Fig. 5) of the initial activity was shown. Using the method of Spande and Witkop [10] we calculated that in the entire enzyme of M_r 220,000, 7 residues were modified; this corresponds to 3.5 residues per enzyme monomer. As the activity had not yet reached 0% it would seem likely that 4 residues on each monomer were involved in enzyme activity. Attempts to protect the enzyme from inactivation with substrate (mannan, mannose and methyl-α-mannoside) were unsuccessful (data not shown).

EXPERIMENTAL

Materials

Routine chemicals and protein modification reagents were obtained from Sigma. Sephacryl S300 and cellulose DE52 were obtained from Pharmacia-LKB.

Enzyme purification

Purification of α-mannosidase was by a modified method of ref. [8] with all stages being performed at 4°. 20 g Jack Bean meal was defatted twice with 200 ml of hexane for 20 min. The meal was dried and stirred with 200 ml 0.05 M Na borate buffer pH 8, centrifuged for 30 min at 10,000 *g* and the supernatant brought to 50% satn with solid $(NH_4)_2SO_4$. The precipitated protein was recovered by centrifugation as before and the ppt resuspended with 10 ml of a 50 mM Na borate buffer pH 8 before dialysis against a 5 mM sol of the same buffer overnight. Dialysis tubing was transferred to 1 l of 0.1 M Tris-HCl pH 8 for 6 h prior to loading onto a Sephacryl S300 gel filtration column equilibrated with 0.1 M Tris-HCl pH 8, 0.25

M NaCl. The column was eluted with the same buffer at a flow rate of 15 ml/h and 5 ml fractions were collected. The enzyme-containing fractions were dialysed against 3 l of 50 mM Tris-HCl pH 8 and loaded onto a cellulose DE52 ion-exchange column equilibrated with 50 mM Tris-HCl pH 8.0. The column was eluted with a gradient of NaCl from 0–350 mM in 600 ml 50 mM Tris-HCl pH 8.0. The column was run at a flow rate of 12 ml/h and 4 ml fractions were collected. The enzyme-containing fractions were run through the DE52 column again under the same conditions and the enzyme-containing fractions were concd to 10 mg/ml in a filter concentrator (Amicon).

Enzyme assay

α-Mannosidase was assayed by the method of ref. [3] using 5 mM *p*-nitrophenyl-α-D-mannopyranoside in 0.05 M NaOAc buffer pH 5, containing 0.1 mM $ZnSO_4$ and 0.1 M NaCl, as a substrate. 100 μl samples of enzyme were incubated with 1 ml substrate for 5 min at 20°, and the reaction stopped by the addition of 2 ml 0.2 M di-Na tetraborate. *A* at 500 nm was measured. One unit of activity was defined as the amount of enzyme releasing 1 mmol of *p*-nitrophenol per min.

Modification of carboxyl groups

Carboxyl groups were modified by the method of ref. [16]. 4 mg of α-mannosidase was incubated at 20° with various H_2O soluble carbodiimides in 50 mM MES/NaOH buffer pH 6.0. EDAC and EDCI were used at concs ranging from 10 mM to 300 mM. At time intervals 10 μl of reaction mixture were added to 40 μl of 100 mM NaOAc buffer, pH 50, and activity was determined as usual.

Modification of tryptophan residues

Tryptophan residues were modified by the method of ref. [15], titrating with increasing amounts of *N*-bromosuccinimide (NBS), in various concs ranging from 0.1–0.6 mM. Aliquots (30 μl) were added to 200 μl of α-mannosidase (4 mg/ml) in 1.5 ml 25 mM NaOAc buffer pH 4.8. The reaction was followed by measuring A_{280}. At various time intervals 30 ml of reaction mixture was added to 120 μl of L-tryptophan (15 mM in 50 mM NaOAc pH 6) and assayed for α-mannosidase activity.

N-*terminal sequencing*

SDS PAGE, native PAGE and electroblotting were prepared and run following the method of ref. [17]. The *N*-terminus of the protein was sequenced from gel-separated proteins by automated Edman sequencing. This was performed as a service by Dr P. Barker, IAPGR Microchemical Facility, Babraham, Cambridge.

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REFERENCES

1. Shepherd, V. and Montgomery, R., *Biochimica Biophysica Acta*, 1976, **429**, 884.
2. Snaith, S. M. and Levvy, G. A., *Biochemistry Journal*, 1968, **110**, 663.
3. Li, Y-T., *Journal of Biological Chemistry*, 1967, **242**, 5474.
4. Einhoff, W. and Rudiger, H., *Biological Chemistry Hoppe-Seyler*, 1988, **369**, 165.
5. McCarter, J. and Withers, S., *Current Opinion in Structural Biology*, 1994, **4**, 885.
6. Vyas, N., *Current Opinion in Structural Biology*, 1991, **1**, 732.
7. Paus, E., *European Journal of Biochemistry*, 1977, **73**, 15510.
8. Sammour, R. H., Gatehouse, J. A., Gilroy, J. and Boulter, D., *Planta*, 1984, **161**, 61.
9. Levy, H. M., Leber, P. D. and Ryan, E. M., *Journal of Biological Chemistry*, 1963, **238**, 3654.
10. Spande, T. F. and Witkop, B., *Methods in Enzymology*, 1967, **11**, 498.
11. Imoto, T., Johnson, L. N., North, A. C. T., Phillips, D. C. and Rupley, J. A., In *Enzymes*, Vol. 7, 3rd edn. ed. P. D. Boyer. Academic Press, New York, 1972, p. 666.
12. Clarke, A. J. and Svensson, B., *Carlsberg Research Communications*, 1984, **49**, 111.
13. Clarke, A. J. and Yaguchi, M., *Biochimica Biophysica Acta*, 1986, **870**, 401.
14. Deshpande, V., Hinge, J. and Rao, M., *Biochimica Biophysica Acta*, 1990, **1041**, 172.
15. Bray, M. R. and Clarke, A. J., *Biochemistry*, 1995, **34**, 2006.
16. Bray, M. R. and Clarke, A. J., *Biochemistry Journal*, 1990, **270**, 91.
17. Sambrook, J., Fritsch, E. F. and Maniatis, T., *Molecular Cloning—A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, 1989.