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CHEMICAL MODIFICATION OF α-GALACTOSIDASE FROM COCONUT

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Key Word Index—*Cocos nucifera*; α-galactosidase; chemical modification; tryptophan; tyrosine; carboxyl; active site.

Abstract—α-Galactosidase from coconut kernel was inhibited by chemical modification of its tyrosine, tryptophan and carboxyl groups. Treatment with *N*-bromosuccinamide and tetranitromethane indicated that modification of one tryptophan and one tyrosine residue inhibited enzyme activity by 55 and 84%, respectively. Modification of carboxyl groups by carbodiimide indicated that inhibition was due to modification of two carboxyl groups. In the presence of the competitive inhibitor D-galactose, α-galactosidase was protected from inhibition by *N*-bromosuccinamide, tetranitromethane and carbodiimide. These results indicate that a tryptophan, tyrosine and two carboxyl groups are at or near the active site of α-galactosidase.

INTRODUCTION

Although α-D-galactosyl linkages are of common occurrence, the α-galactosidases (EC 3.2.1.22) that hydrolyse these linkages have received little attention [1]. α-Galactosidases are commonly found in plant seeds and are involved in the breakdown of α-D-galactosyl oligosaccharides and polysaccharides during germination [2]. In coconut a deficiency of α-galactosidase may prevent germination [3]. A deficiency of α-galactosidase in humans results in Fabry's disease [4]. α-Galactosidase is used in industry to hydrolyse raffinose in beet sugar molasses [5] and the raffinose family of oligosaccharides in soybean milk [6]. Although α-galactosidase is an important enzyme few concrete facts are available regarding the mechanism of its action because of insufficient knowledge of its chemistry and kinetics [1]. Studies on the inhibition of α-galactosidase from sweet almond by metal ions and photooxidation have indicated the involvement of carboxyl groups and histidine residues at its active site [7]. In this paper we describe the results of an investigation of the amino acid residues at the active site of α-galactosidase from coconut (*Cocus nucifera* L.) kernel by chemical modification.

RESULTS AND DISCUSSION

The importance of specific functional groups for the activity of α-galactosidase was determined by the use of reagents with restricted amino acid specificity. The results of the initial inhibition of studies are shown in Table 1. No inhibition was observed when α-galactosidase was reacted with iodoacetamide and iodoacetate, indicating the non-involvement of sulphydryl, imidazole, methionine and amino groups in catalysis [8]. The lack of inhibition of α-galactosidase by diethylpyrocarbonate also indicates that imidazole residues do not take part in catalysis [9]. α-Galactosidase was inhibited by *p*-chloromercuribenzoate but not by *N*-ethylmaleimide. This inhibition by *p*-chloromercuribenzoate was probably due to non-competitive inhibition of α-galactosidase by Hg^{2+} ions [10] and not due to modification of sulphydryl groups [11]. The lack of inhibition of α-galactosidase by 2,4-pentanedione indicates the non-involvement of lysine and arginine residues [12]. α-Galactosidase was inhibited by tetranitromethane, *N*-bromosuccinamide and carbodiimide. The enzyme modified with carbodiimide was not reactivated by treatment with hydroxylamine. This indicated crucial roles for tyrosine, tryptophan and carboxyl groups in catalysis. In view of the above results the involvement of tyrosine and tryptophan and carboxyl residues in catalysis was investigated.

The results show (Fig. 1) that modification of one tryptophan residue inhibited the activity by 55%. In the presence of D-galactose, a competitive inhibitor of α-galactosidase [10], modification of one tryptophan residue resulted in only 7% inhibition. Thus this modified tryptophan residue could not be at the active site. A plot of the number of tryptophan residues oxidized vs. the consumption of *N*-bromosuccinamide (NBS) was linear both in the presence and absence of the competitive inhibitor D-galactose. Thus under these experimental conditions NBS was specific for tryptophan residues. These results show that a tryptophan residue is at or near the active site of α-galactosidase and that this residue is protected from modification with NBS by D-galactose. They also indicate that D-galactose, while protecting the active site, brings about conformational changes that make the tryptophan residues outside the active site more accessible to NBS (Fig. 1).

The reaction of α-galactosidase with tetranitromethane was dependent on the concentration (Fig. 2). Nitration proceeded rapidly at first and then slowed down presumably due to breakdown of the reagent. Tetranitromethane reacts with both cysteinyl and tryosine residues [13]. But the effect of pH on inhibition by tetranitromethane (Fig. 3) showed that no inhibition took place below pH 6, which is the pH range at which

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Dedicated to Dr. Luis F. Leloir on the occasion of his 80th birthday 6 September 1986.

Table 1. Effect of chemical inhibitors on α-galactosidase activity

Chemical	Inhibitor concn in reaction mixture (mM)	Residual activity (%)	Incubation buffer
Iodoacetate	10	100	0.1 M sodium citrate, pH 6.0
		98	0.1 M Tris–HCl, pH 7.5
Iodoacetamide	10	98	0.1 M sodium citrate, pH 6.0
		96	0.1 M Tris–HCl, pH 7.5
Diethylpyrocarbonate	7	100	0.1 M sodium phosphate, pH 6.0
N-Ethylmaleimide	10	96	0.1 M sodium phosphate, pH 7.0
p-Chloromercuribenzoate	0.5	18	0.1 M imidazole–HCl, pH 7.0
2,4-Pentanedione	10	100	0.2 M sodium phosphate, pH 7.0
Carbodiimide	10	83	0.1 M TEMED–HCl, pH 4.5
	20	65	0.1 M TEMED–HCl, pH 4.5
Tetranitromethane	8.4	16	Tris–HCl, pH 8.0
N-Bromosuccinamide	0.05	0	0.1 M sodium acetate, pH 4.0
	0.008	14	0.1 M sodium acetate, pH 4.0

oxidation of sulphydryl groups occurs [13]. Thus the inhibition of α-galactosidase by tetranitromethane was due to modification of tyrosine residues. D-Galactose protected the enzyme from inhibition by tetranitromethane (Fig. 4). So tyrosine residues are at or near the active site of α-galactosidase. Quantitation of tyrosine modification (Fig. 5) showed that modification of a single tyrosine residue resulted in 84% inhibition.

Incubation of α-galactosidase with carbodiimide (20 mM) in the presence of glycine methyl ester at pH 4.5 resulted in 35% inhibition and this modified enzyme when treated with hydroxylamine at pH 7.0 was not reactivated. At acidic pH values, in addition to carboxyl groups, sulphydryl groups [14] and tyrosines [15] can also be modified. Inhibition due to reaction with thiol groups can be ruled out as amino acid analysis shows the absence of free sulphydryl groups in coconut α-galactosidase [16] and the enzyme is not inhibited by *N*-ethylmaleimide (Table 1). Reaction with tyrosine can be ruled out as the enzyme is not reactivated by treatment with hydroxylamine [17]. The semi-logarithmic plot of percentage residual activity against time for inactivation by carbodiimide is linear (Fig. 6). This indicates that the inactivation process exhibits pseudo-first-order kinetics with respect to time at the given inhibitor concentrations. Treatment of the results according to Levy *et al.* [18] by plotting the log of the rate of inactivation (pseudo-first-order rate constant for enzyme inactivation) against the log of inhibitor concentration yields a slope of 1.8 (Fig. 7). This indicates that, on average, two molecules of carboxyl groups per molecule of α-galactosidase are inactivated by carbodiimide. D-Galactose protects the enzyme from

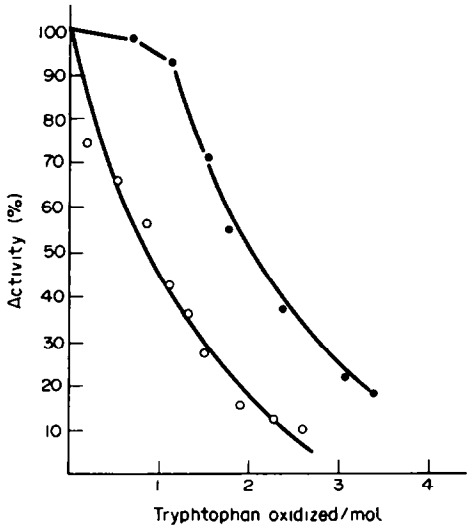

Fig. 1. Inhibition of α-galactosidase by *N*-bromosuccinamide in the absence (○) and presence (●) of the competitive inhibitor D-galactose (100 mM) at pH 4.0

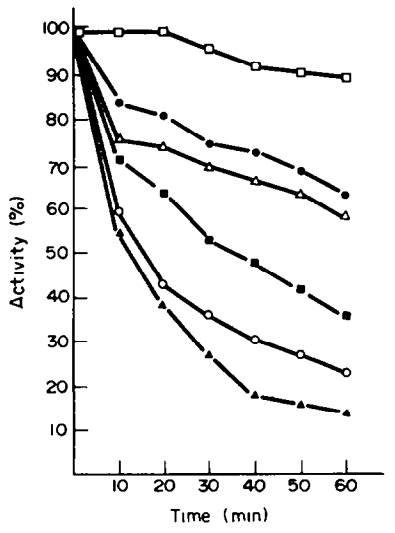

Fig. 2. Effect of the tetranitromethane concentration on the inhibition of α-galactosidase as a function of time. Enzyme incubated with 0 (□), 50 μM (●), 100 μM (△), 250 μM (■), 500 μM (○) and 1000 μM (▲) tetranitromethane in 50 mM Tris–HCl buffer (pH 8.0).

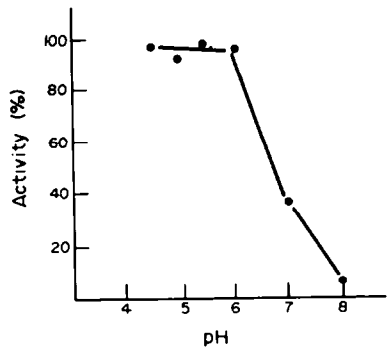

Fig. 3. Effect of pH on the inhibition of α-galactosidase by tetranitromethane.

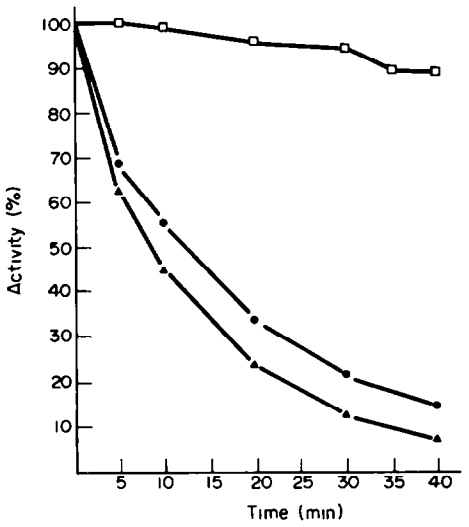

Fig. 4. Effect of the competitive inhibitor D-galactose on the inhibition of α-galactosidase by tetranitromethane. Enzyme incubated in 50 mM Tris–HCl buffer (pH 8.0) with: no inhibitor (□); 500 μM tetranitromethane (▲); 500 μM tetranitromethane and 100 mM D-galactose (●).

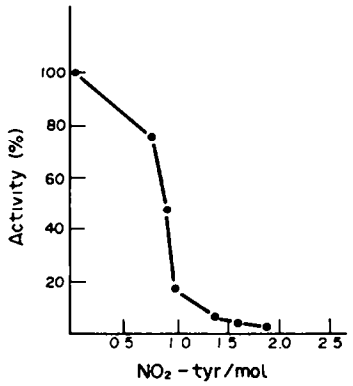

Fig. 5. Correlation of the inactivation of α-galactosidase with the number of tryosines modified. The enzyme (2.2 mg) was incubated with 500 μM tetranitromethane.

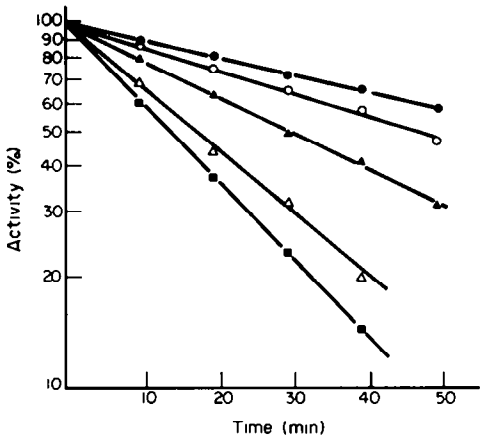

Fig. 6. Effect of carbodiimide (EDC) concentration on the inhibition of α-galactosidase. α-Galactosidase (28 μg) was incubated in 0.1 M TEMED–HCl buffer, pH 4.5, with EDC concentrations of 10 (●), 15 (○), 20 (▲), 25 (△) and 30 mM (■), and glycine methyl ester concentrations of 40, 60, 100 and 120 mM, respectively. The control was incubated under the same conditions without EDC and glycine methyl ester.

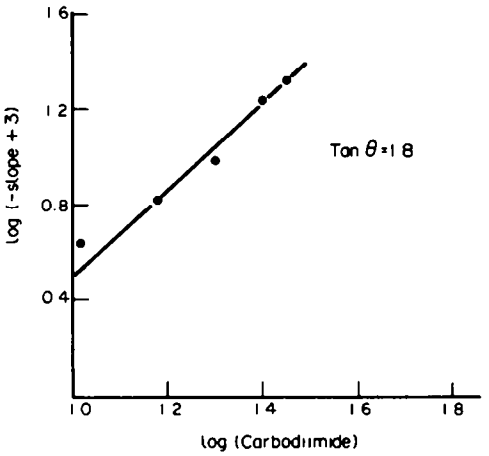

Fig. 7. Kinetic analysis of α-galactosidase inactivation by carbodiimide. The rate of inactivation (slope) obtained from the data of Fig. 6 was plotted in logarithmic form against the log of the carbodiimide concentration.

inhibition by this inhibitor (Fig. 8). These results indicate that two carboxyl groups are at or near the active site.

EXPERIMENTAL

Tetranitromethane was purchased from Aldrich and PD 10 columns were from Pharmacia. All other chemicals were purchased from Sigma.

Purification of α-galactosidase. α-Galactosidase from coconut endosperm was purified by hydrophobic chromatography [16]. The purified enzyme was shown to be homogeneous by isoelectric focusing.

Enzyme assay. This was carried out as described by Dey and Pridham [19] using *p*-nitrophenyl-α-D-galactopyranoside as the substrate.

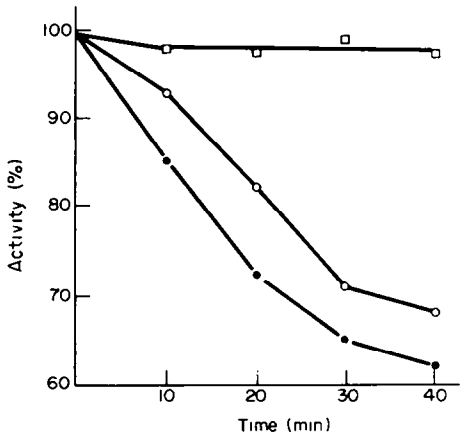

Fig. 8. Effect of the competitive inhibitor D-galactose, on the inhibition of α-galactosidase by carbodiimide. Enzyme incubated in 0.1 M TEMED–HCl buffer, pH 4.5, with no inhibitor (□), with 20 mM EDC and 80 mM glycine methyl ester (●), and with 20 mM EDC, 80 mM glycine methyl ester and 10 mM D-galactose (○).

Reaction of α-galactosidase with specific inhibitors. The effects of a number of specific inhibitors on α-galactosidase activity were tested (Table 1). The enzyme was incubated with the inhibitor at 30° for 30 min and assayed for activity unless stated otherwise. Controls were performed by incubating the enzyme with the appropriate buffer and also by incubating the inhibitor without the enzyme to check for any subsequent interference in the assay. Reactions with *N*-ethylmaleimide, iodoacetate, iodoacetamide and *p*-chloromercuribenzoate were carried out with 0.1 ml α-galactosidase (21 μg), 0.2 ml inhibitor and appropriate buffer to 1 ml. After incubation, 0.1 ml samples were removed and assayed for enzyme activity. In the cases of tetranitromethane and diethylpyrocarbonate 10 μl each and for *N*-bromosuccinamide 50 μl of the inhibitor was used. Reaction with carbodiimide was done by incubating 0.1 ml α-galactosidase (21 μg) with 0.1 ml 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (100 mM), 0.1 ml glycine methyl ester (400 mM) and 0.7 ml TEMED–HCl buffer (pH 4.5). The modified enzyme was dialysed against 0.1 M TEMED–HCl buffer (pH 7.0), incubated with 0.5 M hydroxylamine for 5 hr and tested for enzyme activity. In the case of pentanedione 10 μl of the inhibitor dissolved in EtOH was incubated with the enzyme for 24 hr.

Modification of tryptophan with N-bromosuccinamide. To 1 ml α-galactosidase (0.25 mg) in 0.1 M acetate buffer (pH 4.0) at 20°, 10 μl portions of 1 mM *N*-bromosuccinamide were added and the decrease in absorbance at 280 nm was measured after 5 min. Samples (10 μl) were removed after each addition and diluted in 1 ml 0.1 M McIlvaine buffer [20] (pH 5.5) and assayed for enzyme activity. The number of tryptophan residues oxidized per mol of α-galactosidase was calculated from the decrease in absorbance at 280 nm according to the method of Spande and Witkop [21]

$$n = \frac{\Delta A \times 1.31 \times M_r}{A \times \text{a.f.} \times 5500}$$

where n is the number of tryptophan residues per mol of protein, A is the initial optical density at 280 nm, A is the corrected optical density decrease at 280 nm, a.f. is the absorptivity factor to convert A at 280 nm into mg/ml protein = 0.4, and M_r = 19875 [16]. Inhibition by *N*-bromosuccinamide was also done in the presence of 100 mM D-galactose under the same conditions. The reaction mixture contained 1 ml α-galactosidase (0.25 mg) in 0.1 M acetate buffer (pH 4.0) containing 100 mM D-galactose.

Effect of tetranitromethane concentration. Modification of the tyrosine residues by tetranitromethane was carried out as described by Sokolvsky *et al.* [13]. α-Galactosidase (0.5 ml, 0.12 mg) and 0.5 ml 50 mM Tris–HCl buffer (pH 8.0) were incubated at 30° with 10 μl tetranitromethane suitably diluted in EtOH. The final tetranitromethane concns were varied from 50 to 1000 μM, and 0.1 ml samples were removed at 10 min intervals, diluted 1/10 with 0.1 M McIlvaine buffer (pH 5.5) and assayed for enzyme activity. The controls was incubated with 10 μl EtOH.

Determination of the number of tyrosine residues modified during inhibition. α-Galactosidase (9 ml, 2.2 mg) and 9 ml 50 mM Tris–HCl buffer (pH 8.0) were incubated at 30° for 5 min. The enzyme activity was then assayed after 1/10 dilution with McIlvaine buffer (pH 5.5) and 2 ml of the soln was eluted through a Pharmacia PD 10 column. To the remaining soln was added 160 μl 50 mM tetranitromethane in MeOH. At 10 min intervals the solution was assayed for enzyme activity and 2 ml samples were eluted through a PD 10 column as described above. The enzyme eluted from the PD 10 column was hydrolysed in 6 M HCl for 24 hr at 110° and the hydrolysate subjected to amino acid analysis (Durrum D-500 analyser).

Modification of carboxyl groups by carbodiimide. α-Galactosidase (28 μg) was incubated in 0.1 M TEMED–HCl buffer (pH 4.5) with 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC) and glycine methyl ester at 30°, and at 10 min intervals 0.1 ml samples of the soln were tested for α-galactosidase activity. The final EDC and glycine methyl ester concentrations were varied from 10 to 30 mM and from 40 to 120 mM, respectively.

Effect of D-galactose on inhibition by carbodiimide (EDC). α-Galactosidase (28 μg) was incubated with EDC (15 mM) and glycine methyl ester (60 mM) in 0.1 M TEMED–HCl buffer (pH 4.5) in the presence and absence of 10 mM D-galactose. Enzyme activity was determined at 10 min intervals using 0.1 ml samples. The control was incubated under the same conditions but without EDC.

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