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Effect of chemical modification on struture and activity of glucoamylase from *Aspergillus candidus* and *Rhizopus* species

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Abstract. The histidine, tyrosine, tryptophan and carboxyl groups in the enzyme glucoamylase from *Aspergillus Candidus* and *Rhizopus* species were modified using group specific reagents. Treatment of the enzyme with diethylpyrocarbonate resulted in the modification of 0·3 and 1 histidine residues with only a slight loss in activity (10% and 35%) of glucoamylase from *Aspergillus candidus* and *Rhizopus* species respectively. Modification of tyrosine either by N-acetylimidazole or [I^{125}]-leads to a partial loss of activity. Under denaturing conditions, maltose did not help in protecting the enzyme against tyrosine modification or inactivation. Treatment with 2-Hydroxy-5-nitro benzyl bromide in the presence of urea, photooxidation at pH 9·0, N-bromosuccinamide at pH 4·8 resulted in a complete loss of activity· However, the results of experiments in the presence of maltose and at pH 4·8 photooxidation and N-bromosuccinamide treatment suggested the presence of two tryptophan residues at the active site. There was a complete loss of enzyme activity when 10 and 28 carboxyl groups from *Aspergillus candidus* and *Rhizopus,* respectively were modified. Modification in the presence of substrate maltose, showed at least two carboxyl groups were present at the active site of enzyme and that only one active center seems to be involved in breaking ally 3 types of α-glucosidic linkages namely α-1,4, α-1, 6 and α-l,3.

Keywords. Glucoamylases; chemical modification; structure and activity.

Introduction

Glucoamylase [α-l,4-D glucan glucohydrolase EC 3·2·1·3] catalyses the hydrolysis of starch, producing D-glucose as the main product. Our studies on the structure and stability of glucoamylase II from *Aspergillus niger* suggested that the environment around aromatic aminoacids is critical for activity and binding of the synthetic substrate, and that *p*-nitrophenyl-α-D-glucoside perturbed the environment around aromatic amino acids and caused a decrease in the ordered structure (Shenoy *et al.,* 1984)· Glucoamylases from different fungal sources differ from one another in their physical and chemical properties (Manjunath *et al.,* 1983; Shenoy 1984). In the case of glucoamylase from *A. niger* and *A. saitoi* the presence of tryptophan and carboxyl groups at the active site is implicated (Jolley and Gray, 1976; Inokuchi *et al.,* 1982b; Ohnishi and Hiromi, 1976; Frankel-Conrat, 1957; Barker *et. al.,* 1971; Gray and Jolley, 1973; Hoschke *et al.,* 1976). In addition, tyrosine and histidine residues have been identified at the active site (Zberetkosov *et al.,* 1976; Hoschke *et al.,* 1980a. Investi-

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Abbreviations used: HNBB, 2-Hydroxy-5-nitrobenzyl bromide; NBS, N-bromosucciniamide; BSA, bovine serum albumin; EDC, 1-ethyl-3 (dimethylaminopropyl) carbodiimide; M_r, molecular weight; CD, circular dichroism·

gation have been conducted to ascertain whether these amino acid residues are also present at the active site of the glucoamylases of *A· candidus* and *Rhizopus* species. The tryptophan, tyrosine, histidine and carboxyl groups in glucoamylase from *A· candidus* and *Rhizopus* species were modified with group specific reagents and the effect of such modification on activity and on the secondary and tertiary structure are reported in this communication·

Materials and methods

Glucoamylase from *A· candidus* was a gift from Hindustan Antibiotics Ltd., Pimpri. *Rhizopus* glucoamylase, diethyl pyrocarbonate, N-acetylimidazole, 2-hydroxy-5-nitrobenzyl bromide (HNBB), N-bromosucciniamide (NBS), glycine methyl ester, p-choloromercuribenzoate, iodoacetamide, chloramine-T, N-ethylmaleimide, diisopropyl-fluorophosphate, bovine serum albumin (BSA), maltose, isomaltose and nigerose were from the Sigma Chemical Company, St. Louis, Missouri, USA· Urea and 1-ethyl-3 (dimethylaminopropyl) carbodiimide (EDC) were from Pierce Chemical Company, Rockford, Illinois, USA. Bolton and Hunter reagent [N-succinimidyl-3-(4-hydroxyl-5 [^{125}I] iodophenyl) propionate] and [^{125}I] were Radiochemical Centre, Amersham, England. All other chemicals used were of analytical reagent grade.

Glucoamylase from *A· candidus* and *Rhizopus* species were from purified (Shenoy, 1984) and activity determined using soluble starch as substrate (Manjunath and Raghavendra Rao, 1979). One unit of enzyme activity is defined as that of enzyme which produces one μmol of glucose per min under standard conditions. Specific activity is expressed as units per mg protein. The glucoamylase from *A. candidus* has a moleculor weight (M_r) of 71,000 and specific activity of 436 units*.

Modification of histidyl residues were carried out according to the method of Kumagai *et al.* (1975) using diethyl pyrocarbonate.

Tyrosyl residues were modified according to Riordon *et al.* (1965) by using 400 fold molar excess of N-acetylimidazole. Iodination of the enzyme was carried out using the chloramine-T method (Greenwood and Hunter, 1963). The reaction mixture contained 0·01 ml of enzyme (0·5 mg/ml) 0·01 ml of 0·05 M phosphate buffer, pH 7·5, 0·02 ml of [^{125}I] (1 mCi/20 μl), 0·01 ml of chloramine-T (4 mg/ml). The reaction was carried out for 10 s and stopped with the addition of 0·05ml of metabisulphate (4 mg/ml). Then, 0·01 ml of KI (10mg/ml) was added and the mixture was loaded on a column of sephadex G. 10 (0·9 × 30 cm) and eluted with 0·01 M phosphate buffer pH 7·5 containing 0·15 M saline and 0·5% albumin. The extent of iodination of the protein was calculated from the radioactivity incorporated and the quantity of the enzyme protein and the known specific activity of the iodine. Iodination was also carried out by using iodinated ester of N-succinimidyl 3-(4 hydroxy 5 [^{125}I-]-iodophenyl) propionate according to Bolton and Hunter (1973). Three methods were used for the modification of tryptophan residues. Photooxidation was carried out by the method Barker *et al.* (1971) using 0·001% rose bengal and the tryptophan content of the enzyme was determined by the method of Spies and Chambers (1949). Another method of modification of tryptophan groups was that of Terao and Ukita (1969) using HNBB. The modified enzyme was dialysed against 0·05 M acetate buffer.

*The *Rhizopus* enzyme has a M_r of 55,000 and a specific activity of 142 units (Shenoy, 1984).

pH 4·8 instead of water and its activity tested using soluble starch as substrate. Modification of tryptophan groups was also carried out using NBS according to the method of Ohnishi and Hiromi (1976).

Modification of carboxyl groups was carried out according to the method of Gray and Jolley (1973). For amino acid analysis 500 μl aliquots were withdrawn from the reaction mixture and chromatographed on Biogel P-10 column (0·9 × 60 cm) equilibrated with 0·2 M acetate buffer pH 4·8. Fractions containing protein were pooled, hydrolysed and amino acid content determined (Shenoy, 1984).

Circular dichroism (CD) measurements were made with a JASCO-J20C automatic recording spectropolarimeter calibrated with d-10-camphor sulphonic acid Quartz cells of different path length (1 cm, 0·1 cm) were used for measurements in the region 350–200 nm. Slits were programmed to yield a band width of 10 Å at each wavelength. Mean residue ellipticities $[\theta]_{mrw}$ were calculated by standard procedures (Adler *et al.,* 1973). A value of 110 for mean residue weight was used. The CD spectra were analysed by the method of Provencher and Glockner (1981) to estimate the secondary structure.

Results

Modification of histidine residues

The results of the study of the effect of the treatment with diethyl pyrocarbonate on activity and histidine content of glucoamylases are shown in figure 1. By the procedure adopted only 0·3 and 1 residue could be modified in *A· candidus* and *Rhizopus* glucoamylases, respectively. With the modification of 0·3 residue of histidine around 10% of activity was lost in the case of *A· candidus* glucoamylase where as in the case of *Rhizopus* species around 35% of activity was lost with the modification of one residue in 30 min.

Modification of tyrosine residues

N. acetylimidazole: Acetylation of tyrosine groups in glucoamylase from the two sources was carried out both under non-denaturating conditions and in 8 M urea. In the absence of urea, only one tyrosine residue was modified in case of the *A· candidus* enzyme resulting in a 10% loss of activity. Although 4 tyrosine residues were modified in case of *Rhizopus* glucoamylase, activity loss was only 30%. In the presence of an unfolding reagent like 8 M urea, 5 and 9 tyrosine residues were modified by the reagent in the case of *A· candidus* and *Rhizopus* glucoamylases resulting in a 86% loss of activity in both cases. Substrate maltose did not protect the enzyme against modification by the reagent.

Iodination of enzyme with radioactive I^{125}: Iodination of the glucoamylase from two species led to an incorporation of about 40% and 32% radioactive iodine in case of the enzymes *A· candidus* and *Rhizopus* species, respectively. The iodinated *A. candidus* enzyme retained only 32% of its original activity whereas iodinated enzyme from *Rhizopus* species retained 72% of its activity. Iodination with Bolton and Hunter

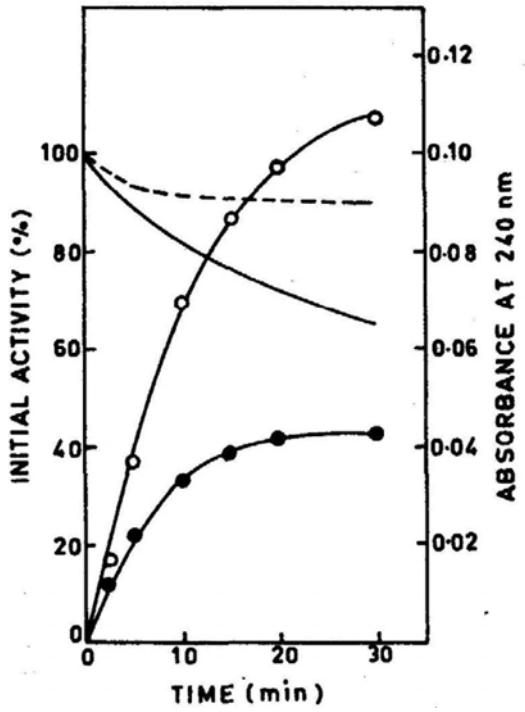


Figure 1. Effect of diethylpyrocarbonate on the enzymic activity and the histidine content of glucoamylases [2·5 ml of enzyme solution (3 mg) in 0·01 M potasium phosphate bluffer pH 6·0, treated with freshly prepared 0·05 ml of diethyl pyrocarbonate (0·08 M in cold ethanol at 6°C for 30 min (control enzyme treated with cold ethanol) E_M = 3200$M^{-1}cm^{-1}$ for carboethoxyl histidyl residues in protein at 242 nm].

(——), Activity of *A· candidus* glucoamylase;(---), activity of *Rhizopus* glucoamylase; (●), Absorbance at 240 nm of *A· candidus* glucoamylase; (O), Absorbance at 240 nm Rhizopus glucoamylase·

reagent was much less effective and resulted in the incorporation of about 4% of radioactivity and retention of 90 ~ 100% of activity·

Modification with NBS

Modification of tryptophan residues with NBS at different pH values resulted in modification to different extents with varying residual activity. At pH 4·8, the pH of optimum activity, treatment of the enzyme with NBS led to the modification of 5 and 4 tryptophan residues from *A· candidus* and *Rhizopus* species respectively and to total loss of activity (figure 2). At this pH, and in the presence of substrate, maltose, only 3 and two tryptophan residues were modified in the glucoamylases of *A. candidus,* and *Rhizopus* respectively and the modified enzymes had 60–65% activity (figure 3). At pH 6·0 only two tryptophan residues were modified by NBS in enzymes from both the species and the modified enzymes of *A· candidus* and *Rhizopus* retained 75 and 65% of its activity, respectively. At pH 7·0 NBS modified only 0·3 and 0·2 residues of tryptophan of the two enzymes and had no effect on the activity·

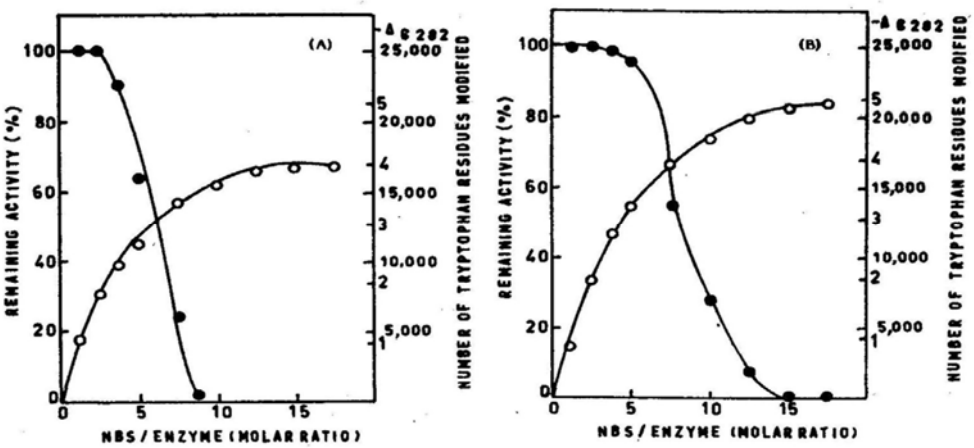


Figure 2. The effect of chemical modification of glucoamylase by NB S at pH 4·8: Enzyme; 8 μM, 0·05 M acetate buffer pH 4·8, 25°C (the number of tryptophan residues modified by NBS was calculated from decrease in the molar difference absorbance coefficient at 282 nm $\in_M$ = 4200). (O), Decrease in molar difference absorption coefficient at 282 nm: (●), the remaining activity of the enzyme· (A) *A. candidus;* (B) *Rhizopus* species.

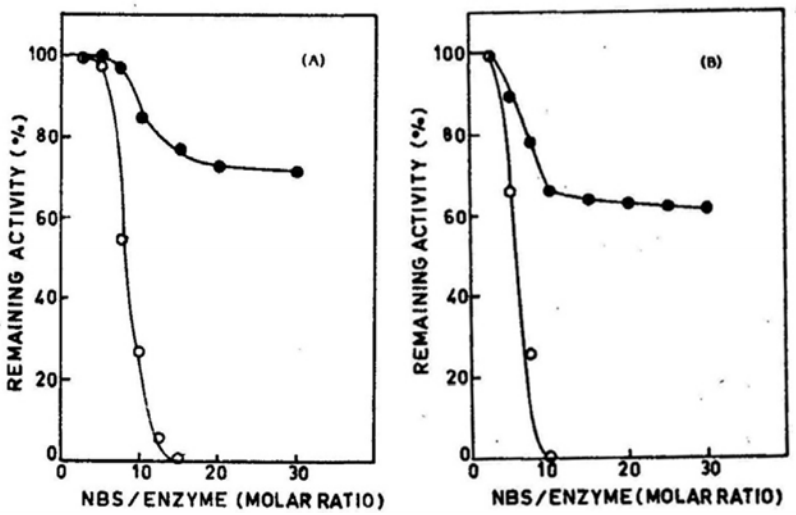


Figure 3. The effect of substrate on the chemical modification by NBS· Chemical modification: Enzyme, 8 μM, 0·05·M acetate buffer, pH 4·8, 25°C.

The remaining activity of the enzyme modified in the absence of substrate maltodextrin (O) and the enzyme modified in the presence of substrate (0·19%) (●) are shown.

(A) *A. candidus*; (B) *Rhizopus* species.

Modification with HNBB

Under non denaturing conditions, 3 and 2 tryptophan residues of the glucoamylase of *A*· *candidus* and *Rhizopus* species respectively were modified· Resulting enzymes

had 60 and 65% of original activity. Under denaturing conditions, *i·e.* in the presence of 8 M urea, 12 and 6 tryptophan residues in the enzymes from *A. candidus* and *Rhizopus* species were modified with a complete loss of activity.

Photooxidation

The results of photooxidation are shown in figure 4. After photooxidation for 30 min at pH 4·8, the enzyme from the two species had 63 and 60% activity with the modification of 3 and 2 tryptophan residues, respectively. In the presence of maltose, the substrate, there was only a 20% loss of activity in enzyme from both the species and only one tryptophan residue was modified. Photooxidation of the enzymes at pH 9·0 resulted in a complete loss of activity within 35 min from both the species. Eight tryptophan residues were modified in case of *A. candidus* and 4 tryptophan residues were modified in case of *Rhizopus* enzyme. Amino acid analysis of the enzyme and also the estimation of tryptophan content of the enzyme showed that only tryptophan groups, and neither histidine nor tyrosine residues were modified by photooxidation (Shenoy, 1984).

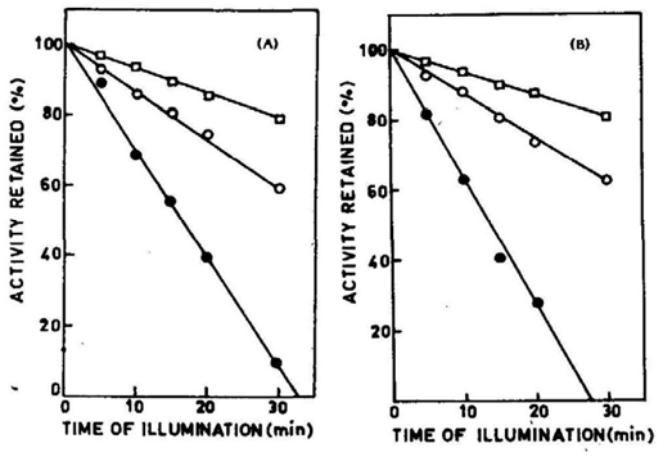
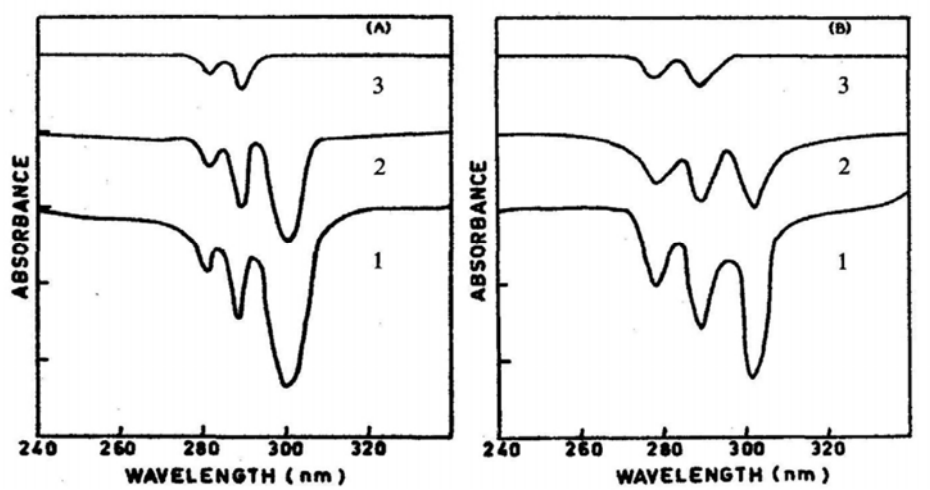


Figure 4. The photooxidation of glucoamylases in the presence of rose Bengal at pH 4·8 (O); pH 9·0 (●), and in the presence of maltose (0·15 M) at pH 4·8 (❑). Solution at each pH value contained glucoamylase (1 mg) and rose Bengal (10 μg) in an initial volume of 11 ml. Maltose (30 mg) was added to 0·6 ml of enzyme die solution immediately prior to photooxidation. The temperature of reaction was 4°C. (A) *A· candidus;* (B) *Rhizopus* species·

UV-difference spectra

To gain a better understanding of the role of aromatic amino acids in glucoamylase, difference-spectral measurements were made in the presence of substrate maltose. The difference spectra (figure 5) in the presence of substrate maltose, are seen with troughs at 281, 289 and 300 nm in case of the *candidus* enzyme and 278, 289 and 302 nm with *Rhizopus* enzyme. The trough near 300 and 302 nm disappeared with



Figured 5. A. Difference spectra produced by maltose on the native and NBS-modified *A·candidus* glucoamylase. Enzyme, 30 μM; NBS (1) O M (native enzyme) (2) 0·27 mM (NBS/enzyme = 8·9). (3) 0·45 mM (NBS/enzyme = 15·0), 005 M acetate buffer, pH 4·8, 25°C.

The difference spectra were measured at pH 4·8 and 25°C with 10 μm glucoamylase and 97 mM maltose·

B. Difference spectra produced by maltose of the intact and NBS-modified *Rhizopus* glucoamylase.

Enzyme 30μM, NBS (1)O M (native enzyme); (2)0·18mM (NBS/enzyme = 6·0) and (3) 0·34 mM (NBS/enzyme = 11·3), 0·05 M acetate buffer, pH 4·8 25°C·

The difference spectra were measured at pH 4·8 and 25°C with 10 μM glucoamylase and 97 mM maltose·

the initiation of modification of the tryptophan groups with NBS followed by a loss in enzyme activity.

Modification of carboxyl groups resulted in a complete loss of activity with in 40 min in both *A. candidus* (figure 6) and *Rhizopus* glucoamylases. However, in the presence of substrate maltose, only 10 and 25% activities were lost in the glucoamylase of *A. candidus* and *Rhizopus* species respectively. In the absence of substrate 10 glycine residues were incorporated into a mol of glucoamylase from *A· candidus* and in the presence of substrate 8 residues were incorporated. 28 and 26 glycine residues were incorporated per mol of glucoamylase from *Rhizopus* species, in the absence and in presence of substrate respectively.

Modification by other group specific reagents

In addition to the above reagents, different reagents specific for free sulphhydryls, serine hydroxyls and divalent metal ions were added separately to the enzyme solution to the requisite concentration. After incubation for 30 min at 6–8°C, the enzyme activity was assayed at 60°C. In enzyme from both the species, 20 mM EDTA, 2 mM *p*-chloromercuribenzoate, 2 mM Iodoacetamide, 2 mM N-ethyl maleimide, 1 mM diisopropyl fluorophosphate did not affect the activity of the glucoamylases from two sources.

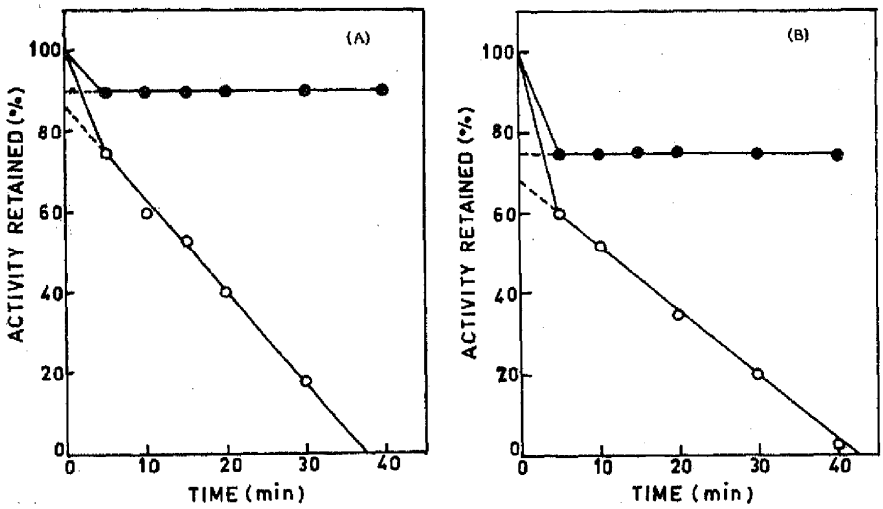


Figure 6. A. Loss of activity of glucoamylase from *A· candidus* on treatment with glycine methyl ester and 1-ethyl-1 (3-dimethylaminopropyl) carbodiimide in absence (O) and in the presence of (●) maltose· The intercepts shown are calculated to be; maltose absent, 86%; maltose present, 90%.

B. Loss of activity of glucoamylase from *Rhizopus* species with glycine methyl ester and 1-ethyl- 1-(3-dimethylaminopropyl) carbodiimide in absence (O) and in presence of maltose (●).The intercepts shown are calculated to be: maltose absent, 68 %; maltose present, 75 %.

In order to know whether there were more than one active center for breaking of 3 types of bonds namely α-1,4, α-1,6 and α-1,3, the carboxyl-rnodified enzyme in the presence of substrate maltose was tested against two other types of substrate, *e·g.* isomaltose and nigerose. Both the substrate were hydrolysed by the enzyme thus showing that the enzyme contains only one active center for the breakdown of all the 3 types of substrates.

Effect of tyrosine modification on structure

The modification of tyrosine residues in the absence of denaturing agent resulted in modification of one tyrosine residue in case of glucoamylase from *A. candidus.* Modification of this residue did not affect near UV CD bands, (except for a slight diminution in the 295 nm band) nor it affected the secondary structure of enzyme.

Effect of Carboxyl groups modification on structure

Modification of carboxyl groups in the absence of substrate resulted in changes in the near UV and far UV CD bands· The CD spectra of the enzyme from *A· candidus* in 0·05 M acetate buffer at pH 4·8 before and after carboxyl group modification is shown in figure 7. In the near UV region, the enzyme exhibited peaks at 304–305, 289, 279, 272, 265 and 257 nm and a trough at 295 nm. After modification, the peaks at 289, 279, 272 nm and trough at 295 nm disappeared. There were new troughs at 282, 276, and 272 nm. The intensity of peak at 304 nm increased. These results

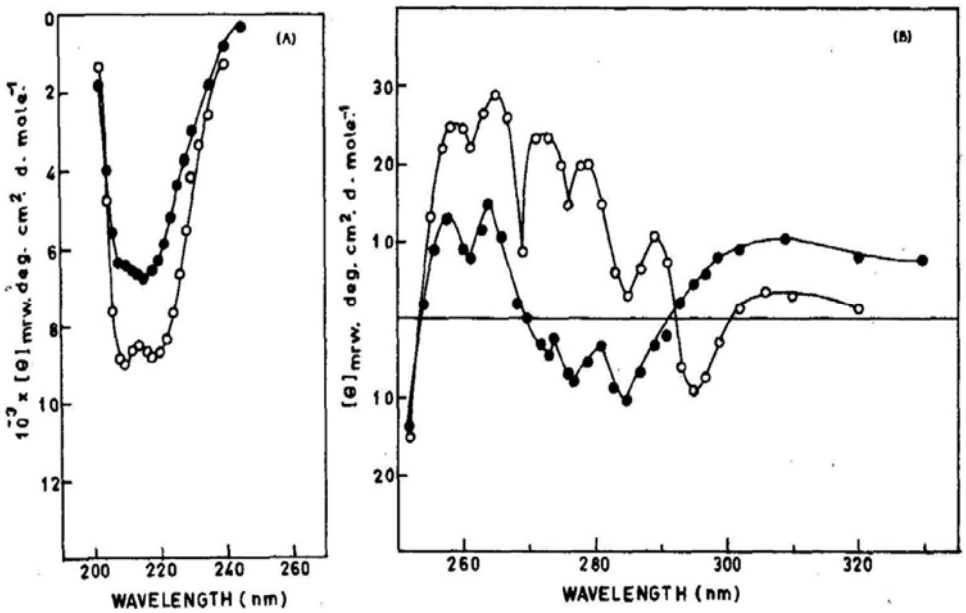


Figure 7. Near UV and far UV CD spectra of glucoamylase from *A. candidus* before and after carboxyl group modification pH 4·8. 0·05 M. Acetate buffer (O) control (●) carboxyl groups modified·

suggest a change in the tertiary structure of enzyme resulting an altered conformation around aromatic aminoacids. In the far UV region, there is a decrease in the ordered structure of enzyme. The analysis of secondary structure of enzyme according to the method of Provencher and Glockner (1981) suggest that the native enzyme has 17% α-helix, 38 % *ß*–structure and 45% aperiodic structure. After modification the helical content decreased to 8%, *ß*-structure 29% and aperiodic structure increases to 63 %. Thus due to carboxyl group modification there was a change in the secondary and tertiary structure of enzyme.

Discussion

The glucoamylase from *A. candidus* species contains 4 histidine residues (table 1). Only a fraction of histidine residues were available for the reagent diethyl pyrocarbonate and the modified enzyme had 90% of its original activity. This implies that histidine groups are not easily accessible for reagent and are not part of active site of the enzyme. The non accessibility of histidine residues could be due to the presence of carbohydrate moiety. Of the total 3 histidine residues of *Rhizopus* enzyme only one residue is modified by the reagent with a 35% loss in activity. Hoschke *et al.* (1980) and Zherebtosov *et al.* (1976) have shown in the case of glucoamylase from *A. niger* modification of histidine residues decreases the binding affinity of the substrate to enzyme and are located away from the catalytic site. Thus it is probable histidine residues are only involved in binding of substrate and are not part of catalytic site·

Glucoamylases from *A. candidus* and *Rhizopus* species contain total of 22 and 23 tyrosine residues, respectively (table 1). In the absence of urea, N-acetyl imidazole modifies only one tyrosine residue from *A. candidus* and 4 from *Rhizopus* species are modified without much detectable change in activity. Modification of tyrosine

Table 1. Effect of group specific reagents on glucoamylases

Reagent	*A. candidus*		*Rhizopus* species	
	Activity	No. of residues modified	Activity	No. of residues modified
Histidine groups modification		4		3
Diethyl pyrocarbonate	90	0·3	65	1
Tyrosine groups modification		22		23
1. N-acetylimidazole				
(a) in absence of urea	85	1	70	4
(b) in presence of urea	14	5	15	9
(c) in presence of urea + substrate	16	5	21	9
2. Iodine 125 (^{125}I)	32 (40)	—	72 (32)	—
Tryptophan groups modification		13		8
1. 2-Hydroxy-5-nitro benzyl bromide				
(a) in absence of urea	60	3	65	2
(b) in presence of urea	0	12	0	6
2. Photooxidation (rose bengal)				
(a) pH 4·8	63	3	60	2
(b) pH 4·8 + substrate	80	1	80	1
(c) pH 9·0	0	8	0	4
3. N-bromosuccinimide				
(a) pH 4·8	0	5	0	4
(b) pH 4·8 + substrate	62	3	66	2
(c) pH 6·0	75	2	65	2
(d) pH 7·0	100	0·3	100	0·2
4. Carboxyl groups modification		46 + 28*		55 + 22*
1. Glycine methyl ester and 1-ethyl-1(3-dimethyl amino-propyl) carbodiimide				
(a) in absence of substrate	0	10	0	28
(b) in presence of substrate	90	8	75	26

*Total number of Asp Gulu residues

residue of glucoamylase from *A. candidus* in the absence of denaturing agent by N-acetylimidiazole did not affect its secondary and tertiary structure. Exposed tyrosine residues are modified by this reagent (Riordan and Valle, 1972) and with the unfolding of enzyme in 8 M urea, all the tyrosine residues are modified with complete loss in activity of the enzyme. It is probable that tyrosine residues which are not exposed to solvent are at the active center and the part of the active center may be in the hydrophobic interior of the molecule. Substrate maltose did not protect the enzyme against modification/inactivation. From our CD measurements in the presence of synthetic substrate *p*-nitrophenyl-α-D-glucoside, in the case of *A. niger* glucoamylase, the intensity of the 277 nm band in near UV CD spectra decreases with increasing concentration of substrate suggesting the possibility of tyrosine being a part of the binding site. With modification, binding ability of substrate might be lost with the resultant decrease in activity. Hoschke *et al.* (1980a,b), have identified tyrosine groups at the active site of the enzyme and showed that binding affinity of the substrate decreased by the modification of tyrosine without any appreciable decrease in catalytic activity. Earlier we had suggested (Shenoy *et al.*, 1984) that the

activity of the enzyme is very sensitive to the environment around aromatic amino-acids in glucoamylases·

Difference spectra measurements conducted in the presence of substrate both before and after modification of tryptophan residues suggest that tryptophan residues are involved in binding of substrate and that due to binding of the substrate the environment around tryptophan changes. The blue shift in the spectrum suggests the transfer of tryptophan groups from a hydrophobic region of high refractive index to the solvent environment of lower polarisibility although rupture of hydrogen bonds cannot be excluded·

Tryptophan residues from the enzyme are modified to difference extent with change in pH. At pH 4·8, the optimum pH of the enzyme, NBS modified 5 tryptophan residues from *A. candidus* and 4 tryptophan residues from *Rhizopus* species with total loss in activity. Lowering the pH from 7·0–4·8, results in a change in conformation of enzyme (Shenoy, B. C., Appu Rao A. G. and Raghavendra Rao, unpublished results) and also the availability of tryptophan residues to NBS· At pH 7·0 enzyme has no activity and only a fraction of tryptophan groups are modified by the reagent These results suggest a direct involvement of tryptophan groups in catalytic activity. In the presence of substrate maltose at pH 4·8 only 3 and 2 tryptophan residues from *A. candidus* and *Rhizopus* species enzyme respectively were modified, while 62 and 66% of activity remained suggesting the protection of tryptophan residues by the substrate. There is a differences in the reactivity of tryptophan residues towards the HNBB reagent under denaturing and non denaturing conditions. Modification under denaturing conditions results in an enzyme with a complete loss of activity. Photooxidation of tryptophan residues suggest the involvement of these residues in catalytic activity.

Carboxyl groups on the enzyme seem to play an important role in catalytic activity. Modification of 10 and 28 of these groups from *A. candidus* and *Rhizopus* enzyme results in a complete loss of activity. In the presence of substrate, two carboxyl groups less are modified in both the enzymes which has retained most of its activity suggesting that at least two carboxyl groups are at the active site. These results are in conformity with the results of Jolley and Gray (1976) and Hiromi *et al.* (1966) have suggested that active form of carboxyl groups at the active site are COO^- and COOH of the enzyme·

Modification of carboxyl groups resulted in a change of secondary and tertiary structure of glucoamylase from *A. candidus.* In case of glucoamylase from *A. saitoi* modification of carboxyl groups with [^{14}C]-l-cyclohexyl-3 (2-morpholinyl (4)-ethyl) carbodimide resulted in complete loss of activity without change in secondary and tertiary structure of enzyme (Inokuchi *et al.,* 1982).

Only one active center seems to be involved in breaking all the 3 types of α-glucosidic linkages namely α-1,4, α-1,6 and α-1,3. An enzyme preparation whose carboxyl groups were modified in the presence of maltose hydrolysed both isomaltose and nigerose completely thus indicating that the glucoamylase contained only one active site for breaking the glucosidic bonds in maltose, isomaltose and nigerose·

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