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Structure and stability of glucoamylase II from *Aspergillus niger:* A circular dichroism study

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Abstract. Glucoamylase II (EC 3.2.1.3) from *Aspergillus niger* has 31 % α-helix, 36 % *β*-structure and rest aperiodic structure at pH 4·8 as analysed by the method of Provencher and Glockner (1981, *Biochemistry,* 20,33). In the near ultra-violet circular dichroism spectrum the enzyme exhibits peaks at 304, 289, 282 and 257 nm and troughs at 285, 277 and 265 nm respectively. The enzyme activity and structure showed greater stability at pH 4·8 than at pH 7·0, were highly sensitive to alkaline pH but less sensitive to acid pH values. The enzyme retained most of its catalytic activity and structure even on partial removal of carbohydrate moieties by periodate treatment but was less stable at higher temperatures and storage at 30°C. Reduction of the periodate treated enzyme did not reverse the loss of stability. Binding of the synthetic substrate,*p*-nitrophenyl-α-D-glucoside, perturbed the environment around aromatic amino acids and caused a decrease in the ordered structure.

Keywords. *Aspergillus niger;* glucoamylase; circular dichroism; carbohydrate moieties.

Introduction

Several glucoamylases (α-1,4 glucan glucohydrolase, EC 3.2.1.3) were purified to homogeneity from different fungal sources by us and some of their biochemical and biophysical characteristics were reported (Manjunath and Raghavendra Rao, 1979). Among these enzymes, glucoamylase II, which is the major component from *Aspergillus niger* is a glycoprotein with 18% carbohydrate and with pH and temperature optima of 4·8 and 60°C, respectively. Very little information is available on the structure-function relationships in these glucoamylases. As a part of our ongoing programme of investigations on glucoamylases, we report in this communication the results of our studies on the conformation and stability of the enzyme using near and far ultra-violet (UV) circular dichroism (CD) and activity measurements of the enzyme as a function of pH, temperature and substrate concentration. The effects of a partial removal of the carbohydrate component by periodate oxidation and subsequent reduction either with sodium borohydride or with hydrogen in the presence of platinum oxide on the physical and catalytic properties are also presented here.

Abbreviations used: UV, Ultra-violet; CD, circular dichroism; BSA, bovine serum albumin; hCG, human chorionic gonadotropin.

Materials and methods

Glucozyme a preparation of glucoamylase from *A. niger* was a gift from Anil Starch Products, Ahmedabad. Sodium metaperiodate, *p*-nitrophenyl-α-D-glucoside and bovine serum albumin (BSA) were from Sigma Chemical Co., St. Louis, Missouri, USA. Guanidine HCl was from Pierce Chemical Co., Rockford, Illinois, USA. All other reagents and chemicals used were of analytical grade.

Glucoamylase II from *A. niger* was purified to homogeneity from glucozyme by the method of Manjunath and Raghavendra Rao (1979). Enzyme activity was determined using a 2 % (w/v) starch solution. One unit of enzyme activity is defined as the amount of enzyme required for the liberation of 1 μmol of glucose per min at 60°C. Enzyme activity was also determined using the synthetic substrate *p*-nitrophenyl-α-D-glucoside (Sternberg, 1970).

Protein was determined both by the method of Lowry *et al.* (1951) using BSA as the standard and also from the absorbance of the solution at 280 nm using a value of $A_{lcm}^{1\%} = 13{\cdot}8$. This value was obtained by measuring the absorbance of a series of solutions whose concentrations were determined by the microKjeldahl procedure for the estimation of nitrogen. A value of 6·25 was used for converting nitrogen to protein. Total carbohydrate was estimated by the phenol-sulphuric acid reaction (Dubois *et al*., 1956). Half-cystine content was determined as cysteic acid after performic acid oxidation according to Moore (1963). Total free – SH groups were estimated by the method of Ellman (1959). The total amino groups was estimated by the method of Habeeb (1966) using 2,4,6-trinitrobenzenesulphonate. Tryptophan content of the enzyme was determined spectrophotometrically according to Edelhoch (1967) in 6 M guanidine HC1. The total aldehyde moieties which were produced by the oxidation of the enzyme with periodate were estimated by the cyanometric method of Dyer (1956).

Periodate oxidation was carried out according to Yasuda *et al.* (1971). Appropriate samples of the enzyme (4 mg/ml) were incubated at room temperature in the dark with 0·1 M sodium metaperiodate and in 0·05 M acetate buffer, pH 4·8. After 30 min the reaction was stopped by the addition of a 5 molar excess of thioglycolate. The enzyme was then recoverd after extensive dialysis at 4°C against 0·05 M acetate buffer, pH 4·8.

Two methods ofreduction of the periodate treated enzyme were tested. The enzyme was reduced with $NaBH_4$ by a modification of the procedure described by Carlson 1966). Freeze-dried enzyme (1 mg/ml) in 0·1M phosphate buffer, pH 7·0 was treated with 0·3 M $NaBH_4$ at 37°C and the pH was maintained at 7·0 using 1M HC1. After 50 h, the excess of borohydride was destroyed in the cold by the addition of glacial acetic acid to pH 5·0 and the solution was dialysed overnight against 0·05 M acetate buffer pH 4·8 at 4°C with four changes.

The enzyme was reduced with platinum oxide and hydrogen according to Huennekens *et al.* (1963). Platinum oxide (20 mg) was suspended in 2·5 ml of 0·05 M acetate buffer, pH 4·8 in a hydrogenation flask. The catalyst was reduced at 25°C and at atmospheric pressure until no more hydrogen was absorbed. The enzyme (10mg) suspended in 2·5 ml of acetate buffer, pH 4·8 was introduced into the hydrogenation flask. After 4 to 5 h of hydrogenation, the apparatus was flushed with nitrogen. The reduced enzyme was then filtered to remove the catalyst.

CD measurements

CD measurements were made in a JASCO-J-20C automatic recording spectropolarimeter calibrated with d-10-camphor sulphonic acid. Quartz cells of different path length (1 cm, 0·1 cm or 0·05cm) were used for measurements in the region 350 to 200 nm. Slits were programmed to yield a band width of 10Å 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.

For making measurements at different temperatures, the solution was heated to the desired temperature by circulating water through a double walled cell holder from a preheated water-bath and then allowed to remain at that temperature for 15 to 20 min for attainment of thermal equilibrium before making measurements. Concentrations of protein solutions were adjusted to give 0·2–1·5 absorbance at 280 nm in 10 mm light path quartz cells. All the measurements were made in triplicate and at room temperature unless mentioned otherwise.

Results and Discussion

Conformation and storage stability of glucoamylase

Effect of pH: The CD spectrum of the enzyme in 0·05 M acetate buffer at pH 4·8, which is the optimum pH for the activity of the enzyme, is shown in figure 1. In the near UV region the enzyme exhibited peaks at 304,289,282,265 and 257 nm and troughs at 285, 277 and 268 nm (figure 1 A). Chemical and spectrophotometric assays showed that the enzyme contained 13 tryptophan, 14 tyrosine, 19 phenylalanine and 8 half-cystine residues per mol of the enzyme (Shenoy, B.C., unpublished results; Manjunath and Raghvendra Rao, 1979). The bands at 289, 285, 282 and 277 are probably due to tryptophan and tyrosine. Individual assignment of the bands is difficult because of the overlapping nature of tyrosine and tryptophan absorption bands. The bands at 259,265 and 267 nm are due to phenylalanine and that at 304 nm may also possibly be due to tryptophan. The overlapping contribution of cystine bands in this region cannot be ignored (Strickland, 1974). The fine spectral features of the enzyme was retained even after storing the enzyme at 4°C for 10 days at pH 4·8 (figure 1A) indicating little or no conformational changes around the aromatic amino acid residues in the enzyme. The enzyme activity remained unaltered (table 1).

The far UV CD spectrum of the enzyme at pH 4·8 in 0·05 M acetate buffer is shown in figure 1 B. The enzyme has minima at 218 nm and at 210 nm. Analysis of the secondary structure of the enzyme by the method of Provencher and Glockner (1981) indicated that the enzyme contained 31% α-helix, 36% *ß*-structure and 33% remainder (table 1). The figure 1 C shows the computer fit data of the enzyme by the Provencher and Glockner (1981) method. By the curve-fitting procedure of Greenfield and Fasman (1969) it was possible to fit the experimental data fairly well with the assumed value of 15 % α-helix, 40% *ß*-structure and rest aperiodic. Compared to the value obtained by the method of Provencher and Glockner (1981), helical content appears considerably low. For further comparison, only the method of Provencher and Glockner (1981) was

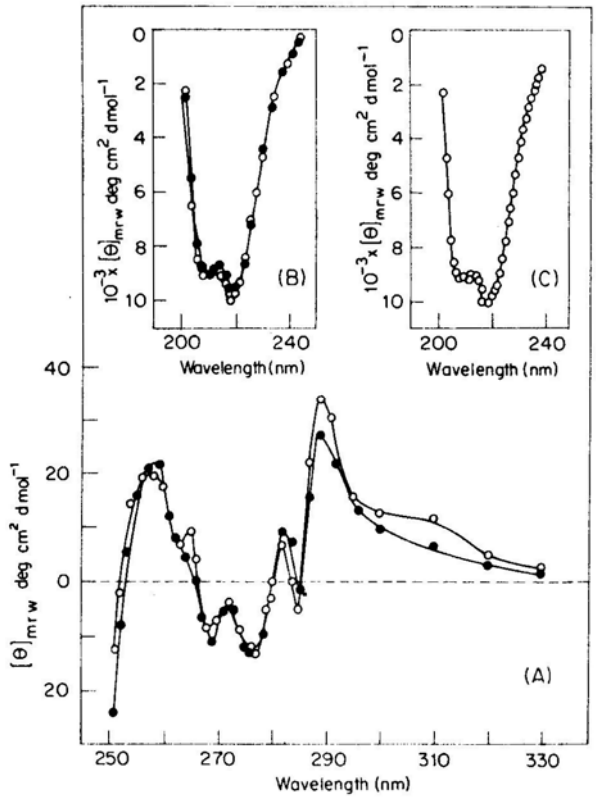

Figure 1. **A.** Near UV CD spectrum of glucoamylase II at pH 4·8. (O), Native (fresh); (n), native after 10 days. **B.** Far UV CD spectrum of glucoamylase II at pH 4·8. (O), Native (fresh); (n), native after 10 days. **C.** Far UV CD spectrum of glucoamylase II.(O), Experimental;(—), calculated (by curve fitting procedure of Provencher and Glockner, 1981).

used in view of the fact that the estimation of the helix and *ß*-sheet by the method of Provencher and Glockner is more accurate than by other methods. After storing the native enzyme at 4°C for 10 days at pH 4·8, there was a decrease in the helical content of the enzyme from 31% α-helix to 22% α-helix and an increase in the *ß*-structure from 36 % to 55 % without any loss of activity (figure 1B).

The near UV CD spectrum of the enzyme at pH 7·0 in 0·016 M phosphate buffer is shown in figure 2A. Compared with the spectrum of the enzyme at pH 4·8, the 282 nm band is absent, the intensity of the 290 nm band was much less and the intensities of the bands at 285, 277 and 269–70 nm increased with the appearance of a new band at 274 nm. The near UV CD spectrum indicated a change in the environment of aromatic amino acids due to an increase in the pH from 4·8 to 7·0. At pH 7·0, the enzyme has only 2 % of its activity at pH 4·8. But when the pH is readjusted to 4·8, the enzyme regained all the activity indicating that the changes in conformation was probably reversible. On storing the enzyme for 10 days at pH 7·0, no significant changes were noticed in the near UV CD spectrum and the activity of the enzyme (assayed at pH 4·8) decreased to about

Table 1. Effect of various physical and chemical treatments on conformation and activity.

	Helical content*			Activity
	α	β	Random	(%)
Native enzyme (pH 4·8, 60°C)				
Native (fresh)	31	36	33	100
After 10 days	22	55	23	100
pH (60°C)				
pH 2·0	22	60	18	61
pH 7·0 (fresh)	24	52	24	2
pH 7·0 (after 10 days)	41	34	25	2
pH 10·0	17	38	45	0
Temperature (pH 4·8)				
15·5°C	20	43	37	0
30·5°C	28	36	36	22
60°C	25	34	41	100
Heat treatment (pH 4·8, 60°C)				
Heat treated at pH 4·8 (60°C, 20 min)	20	47	33	60
Periodate treatment (pH 4·8, 60°C)				
Periodate treated (fresh)	27	24	49	60
Periodate treated (after 10 days)	17	27	56	0
Periodate + heat treated at pH 4·8 (60°C, 20 min)	3	49	48	0
Substrate (pH 4·8, 60°C)	27	41	32	100
Enzyme + *p*-nitrophenyl-α-D-glucoside	26	38	36	100

Activities of the enzyme were determined at pHs and temperatures mentioned. Substrate in all cases except 6 was soluble starch. Details in text.
* By the method of Provencher and Glockner (1981).

87% of the original. The far UV spectrum of the enzyme (figure 2B) suggests an increase in the helical content of the enzyme on storage at pH 7·0 for a period of 10 days (table 1). Although there was an increase in the helical content of the enzyme upon storage (at pH 7·0), activity decreased as mentioned above.

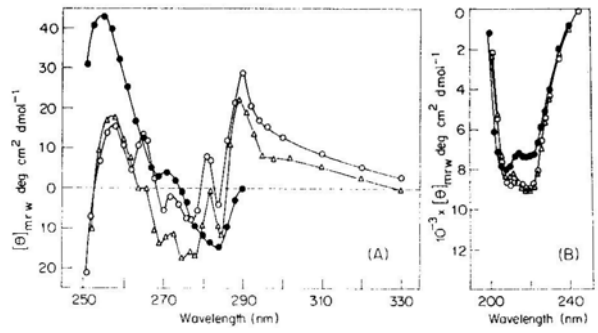

Figure 2. **A.** Near UV CD spectrum of glucoamylase II at various pH values. (O), pH 2·0; (Δ), pH 7·0; (1), pH 10·0. **B.** Far UV CD spectrum of glucoamylase II at various pH values. (O) pH 2·0;(Δ),pH 7·0;(1), pH 10·0.

When the pH of the enzyme solution was raised to 10·0, changes in near UV CD bands (figure 2A) were observed which reflects changes in the conformation of the enzyme. The intensities and position of the band at 290, 282,276 and 258 nm change remarkably indicating the possible ionisation of the tyrosine residues or changes in the environment around aromatic amino acids. The far UV CD spectrum (figure 2B) suggested that there was a progressive loss of secondary structure upon increasing the pH (table 1). The enzyme activity was completely and irreversibly lost at pH 10·0. At pH 2·0, the enzyme still retained 60% of its activity (table 1). The CD spectrum in both far UV and near UV CD (figure 2) suggested that the enzyme was fairly stable at acid pH.

Effect of temperature

In the near UV CD spectrum of the enzyme in the temperature range 15·5°C–60°C, the only change at pH 4·8 was the disappearance of 285 nm band at 15·5°C. The far UV CD spectrum showed that there was a decrease in helical content of the enzyme at 15·5°C and also a slight decrease at 60°C (table 1). This remarkable stability towards heat may possibly be due to a combination of low pH (4·8) and carbohydrate moiety. The other factors which impart thermal stability to protein molecules, such as hydrophobicity (Mozhev and Martinek, 1984) and abundance of disulphide bonds as in RNase are not likely to be significant in this instance. As this enzyme has very few disulphide linkages and secondly even though hydrophobicity increases on removal of the carbohydrate moieties, the stability of the enzyme towards heat and storage is reduced significantly.

Effect of partial removal of carbohydrate moieties

The native enzyme contains 18% total carbohydrates as estimated by Dubois *et al.* (1956) method. After periodate treatment, the carbohydrate content of the enzyme was reduced to 4·5 % (table 1). The enzyme after partial removal of carbohydrate retained most of the near UV CD bands (figure 3A) intact with only a slight decrease in the intensity of the bands indicating that removal of carbohydrate moieties did not significantly change the conformation around aromatic amino acids. The periodate

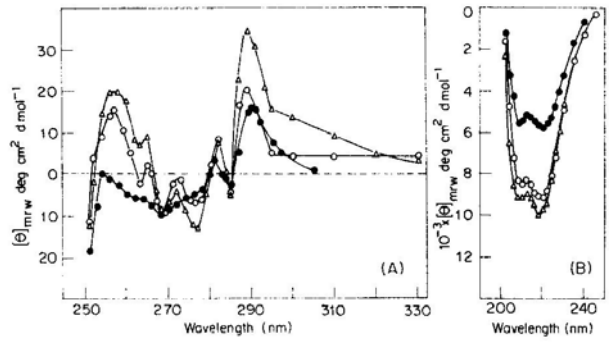

Figure 3. **A.** Near UV CD spectrum ofglucoamylase II. (Δ), Control; (O), periodate treated (fresh); (l), periodate treated after 10 days. **B.** Far UV CD spectrum of glucoamylase II. (Δ), Control; (O), periodate treated (fresh); (l), periodate treated after 10 days.

treated enzyme retained 60% of the original activity. However, the storage stability of such an enzyme preparation to storage was poor since complete loss of activity occurred at 4°C in 10 days. In the near UV region, 276, 265 and 258 nm bands were absent in the spectrum of the periodate treated enzyme. Also the intensity of 282 and 289 nm bands were decreased. These results indicate that the environment around the aromatic amino acids changes considerably after storage of the deglycosylated enzyme.

Immediately after removal of the carbohydrate moiety there was no significant change in the secondary structure of the enzyme at pH 4.8 (figure 3B). But after storing at pH 4·8 for 10 days at 4°C, the enzyme after partial removal of carbohydrate lost considerable amount of the secondary structure which was also reflected in the near UV CD spectrum of the enzyme. The α-helix content decreased from 31 % to 17 %. Activity loss at this stage was almost complete. Thus it appears that the carbohydrate moiety plays an important role in stabilizing the enzyme conformation. It should be mentioned here that periodate treatment did not lead to any change in the aminoacid content of the enzyme. The contents of tryptophan, tyrosine, methionine and half-cystine residues remained the same (Shenoy, B. C. unpublished results).

There was no change in the number of amino groups (8 in the untreated enzyme) immediately after treatment of the enzyme with periodate when 48 aldehyde groups were formed. But on storage for 10 days the amino groups were reduced to 4 from 8 along with the reduction in the aldehyde groups from 48 to 44. No free -SH groups were detected. Gross changes in secondary structure were brought about perhaps by the cross-linking of ε-amino groups of lysine with the aldehyde groups resulting in a total loss of activity (table 2).

Table 2. Effect of periodate oxidation and reduction in presence of hydrogen and platinum oxide (as catalyst).

	Helical content			CH_2O (%)	Aldehyde groups mols/mol	Amino groups mols/ mol	SH groups mols/ mol	Activity (%)
	α	β	Random					
Native enzyme								
Native (fresh)	31	36	33	18	Nil	8	Nil	100
After 10 days	22	55	23	18	Nil	8	Nil	100
Periodate treatment								
Periodate treated (fresh)	27	24	49	4·5	48	8	Nil	60
After 10 days	17	27	56	4·5	44	4	Nil	0
Reduced in presence of hydrogen and platinum oxide								
Fresh	6	44	50	18	Nil	8	Nil	142
After 10 days	6	44	50	18	Nil	8	Nil	142
Periodate + reduced in presence of hydrogen and platinum oxide								
Fresh	5	45	50	4·5	Nil	8	Nil	75
After 10 days	5	45	50	4·5	Nil	8	Nil	70

The effect of heating the native enzyme and the enzyme after partial removal of carbohydrate at 60°C for 20 min (removal of carbohydrate moieties by periodate treatment and the effect of heat on the periodate treated enzyme) on the CD spectrum in both near UV and far UV region in 0·05 M acetate buffer, pH 4·8 are shown in figure 4A and 4B. From figure 4, it is clear that heating (or partial removal of carbohydrate) leads to decrease in the helical content of the enzyme in far UV CD at pH 4·8. But there was a considerable (about 40 %) decrease in the activity of the enzyme (table 1) which was reflected in the far UV CD band and in near UV CD band intensities. The change in intensity of the band at 276 nm and 257 nm indicated some change in conformation of the enzyme. The combined effect of periodate and heat treatment, caused gross conformational changes in the enzyme and leading to a complete loss of activity (table 1). Many enzymes are known to be glycoproteins (Spiro, 1973). Glucoamylases of fungi, studied so far, seem to belong to this category (Manjunath and Raghavendra Rao, 1979; Pazur *et al.,* 1963). Carbohydrate moieties in glycoproteins are known to confer generally resistance to proteolysis (Hayashida, 1975; Hayashida and Yoshino, 1978; Yoshino and Hayashida, 1978), loss of activity upon storage and confer structural stability in many instances against heat and pH, *viz.,* glucoamylase of *A. niger* (Manjunath *et al.,* 1983; Pazur *et al.,* 1970) invertase of *Neurospora crassa* (Gascon *et al.,* 1968) cellulases of a thermophilic fungus (Hayashida and Yoshioka, 1980). In contrast to these observations, removal of carbohydrate moieties resulted in an increase in the thermal stability of human chorionic gonadotropin (hCG) perhaps by increasing the hydrophobicity of hCG (Manjunath and Sairam, 1983). Therefore, it is reasonable to conclude from the results presented that the carbohydrate moieties apparently confer conformational/structural stability towards heat and pH in the present instance.

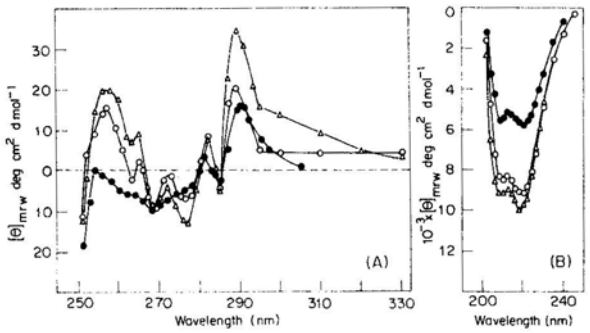

Figure 4. A. Near UVCD spectrum of glucoamylase II,(Δ), Control; (O), heat treated; (l), periodate and heat treated. **B.** Far UV CD spectrum of glucoamylase II. (Δ), Control;(O),heat treated; (l), periodate and heat treated.

Effect of reduction

It is probable that the aldehyde moieties produced by periodate oxidation react during storage with the free amino groups (ε-amino groups of lysine) in the polypeptide leading to loss of conformation and activity. In order to ascertain whether reduction of the aldehyde moieties to primary alcohol groups would improve the stability of the enzyme to heat/storage, the oxidized enzyme was reduced with sodium borohydride at

pH 7·0 for 50 h. But on reduction with sodium borohydride, the enzyme lost its activity completely. Hence a milder reducing agent, hydrogen in the presence of platinum oxide was used to reduce the oxidized enzyme. This enzyme was stable for a period of 10 days but lost its activity completely after a month. No free -SH groups were found in oxidized enzyme after reduction. Activity of the reduced control as well as reduced periodate-treated enzyme was higher than that of the respective controls (table 2). At this stage, it is not possible to explain the cause of this increase in activity.

On reduction of the control enzyme with hydrogen in the presence of platinum oxide as catalyst, there are changes in the near UV CD bands (figure 5A) which reflect changes in the conformation of the enzyme. The band intensities at 258, 290 nm changed remarkably with the disappearance of 265 and 282 nm bands. Activity of the enzyme increased to 142 % of the control. Gross changes in helical structure are seen (figure 5B). On reduction of the periodate treated enzyme the band intensities at 258, 269 and 289 nm change were markedly accompained by a disappearance of 282 nm band. Helical structure was also destroyed completely (figure 5B). No free -SH groups could be detected. Enzyme activity was lost completely after a month, but not in 10 days. The reduction of the oxidized enzyme only helped to prolong the storge stability, but like the oxidized enzyme, it is unstable at higher temperatures. It appears that carbohydrate groups may be necessary for maintaining native conformation of the enzyme.

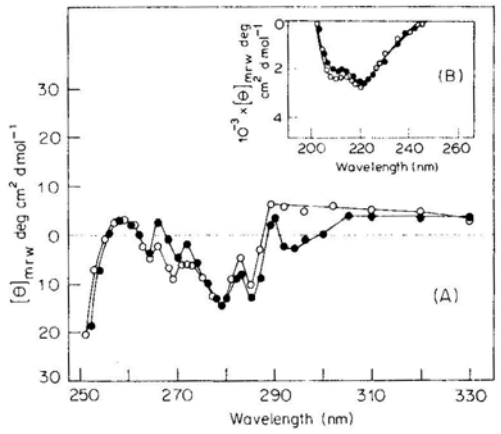

Figure 5. A. Near UV CD spectrum of glucoamylase II. (O), Native enzyme reduced; (l), periodate treated and reduced. **B.** Far UV CD spectrum of glucoamylase II. (O), Native enzyme reduced; (l), periodate treated and reduced.

Effect of substrate

The effect of a synthetic substrate *p*-nitrophenyl-α-D-glucoside on the CD spectrum of the enzyme is shown in figure 6. Although the effects at different concentrations of substrate were measured, only the data at the highest concentration (0·3 mM) is given. The lowest concentration which could be used was 0·1 M, the optical activity of the material determining this limit. *p*-Nitrophenyl-α-D-glucoside has CD bands in both

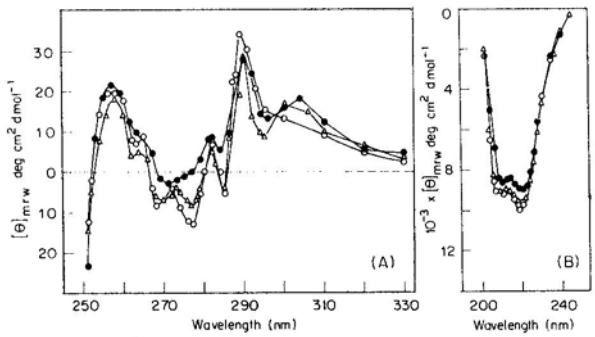

Figure 6. **A.** Near UV CD spectrum of glucoamylase II in the presence of substrate. (O), Control; (Δ), enzyme + substrate ($1 \cdot 10^{-4}$M concentration); (⌊), enzyme + substrate ($3 \cdot 10^{-4}$M concentration). **B.** Far UV CD spectrum of glucoamylase II in the presence of substrate. (O), Control; (Δ), enzyme + substrate ($1 \cdot 10^{-4}$M concentration); (⌊), enzyme + substrate ($3 \cdot 10^{-4}$M concentration).

near UV (300 nm) and far UV (220 nm), but the reaction products D-glucose and *p*-nitrophenol have none in these regions. Appropriate corrections were made for the optical activity of the substrate. From the figure 6A it is clear that, particularly in the presence of the substrate, the bands at 276 and 285 nm are affected considerably. It appears that substrate binding probably occurs at tryptophan and/or tyrosine residues which alters the environment around the tryptophan/tyrosine residues.

Earlier Freedberg *et al.* (1975) reported the isolation and characterization of an exo — 1,4-glucosidase from *A. niger,* with a molecular weight of 62,000. CD measurements of the enzyme revealed 15–25 % α-helixin addition to *β*-structure and random coil. When the enzyme reacts with either synthetic substrate or inhibitor, there is a small but significant decrease in secondary structure (Freedberg *et al.* 1975). Our results are in conformity with those of Freedberg *et al.* (1975).

It could be concluded that the 18 % carbohydrate content of the glucoamylase from *A. niger* may have an important function in stabilizing the conformation/structure of the enzyme as well in protecting the enzyme against loss of activity upon storage or due to changes in the solvent environment.

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