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Fungal glucoamylases

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Abstract. The purification and properties of glucoamylase (α-l,4-glucan glucohydrolase, EC 3.2.1.3) from different fungal sources have been compared. The studies on the conformation and activity of the native enzyme at a function of pH, temperature, substrate concentration and the effect of denaturants and on the role of carbohydrate moiety on structure and stability have been reviewed. The chemical modification of the active centre, binding kinetics of the substrate and active site and the mechanism of action have been summarized. They differ in their fine structure as revealed by their near ultra-violet circular dichroism spectra and contain 30–35 % α-helix, 24–36 % *β*-structure and the rest aperiodic structure. The activity of the enzyme is very sensitive to the environment around aromatic aminoacid residues.

The glucoamylases are glycoprotein in nature, differ in their content and nature of carbohydrate from different sources. The carbohydrate moiety plays an important role in stabilising the native conformation of the enzyme and is not involved in activity and antigenecity.

At the active site of the enzyme, two tryptophan and two carboxyl (glutamate or aspartate) groups are present. It is likely that the histidine and tyrosine residues which are present away from the active site are involved in binding of the substrate. There seems to be seven subsites which are involved in binding of the substrate and the catalytic site is situated in between 1 and 2 subsites. In breaking of α-1,4-, α-1,3-, and α-l,6-bonds only one active centre is involved.

Studies on the immobilization of either glucoamylase alone or as a part of a multienzyme system have been reviewed briefly.

Keywords. Glucoamylases; glycoprotein; circular dichroism; immobilization.

Fungal glucoamylases

Glucoamylase (α-l,4-glucan glucohydrolase, EC 3.2.1.3) is an exoamylase like *β*-amylase but removes a single terminal glucose unit from the non-reducing end of the substrates with the inversion of configuration. It hydrolyses α-1,4-, α-1,6- and also α-1,3- glucosidic bonds (Thoma *et al.,* 1971; Phillips and Caldwell, 1951; Pazur and Kleppe, 1962; Pazur *et al.,* 1962; Kerr *et al.,* 1951). A review on certain aspects of fungal glucoamylase appeared recently (Manjunath *et al.,* 1983) and covered many aspects such as the distribution, assay and purification, physical properties, chemical characteristics, specificity of action and immunochemical analysis. This review proposes to

Abbreviations used: UV, Ultra-violet; CD, circular dichroism; GM_1, glucoamylase I; G M_2, glucoamylase II; IR, infrared; NBS, N-bromosuccinimide; HNBB, 2-hydroxy-5-nitrobenzyl bromide.

discuss some recent work including other aspects such as secondary and tertiary structure and chemical modification *vis-à-vis.* activity.

Purification and properties

Purification

Two glucoamylases from *A. candidus* and *Rhizopus* species were recently purified to homogenity in our laboratory (unpublished results).

Properties

Only one form of enzyme was found in these cases unlike from most other sources (Manjunath and Raghavendra Rao, 1979; Fleming and Stone, 1965; Alazard and Baldensperger, 1982; Takahashi *et al.,* 1981; Ueda and Kano, 1975; Lineback *et al.,* 1969; Watanabe and Fukimbara, 1965; Venkatramu *et al.,* 1975; Svenson *et al.,* 1982). The specific activity of the purified *A. candidus* glucoamylase was 436 which is three times higher than that of the parent strain already reported (Manjunath and Raghavendra Rao, 1979). *A. candidus* and *Rhizopus* glucoamylases were digested to small molecular weight peptides (~ 6000) when treated with pronase whereas that from *A. niger* gave two peptides (60,000 and 12,000), of which the larger peptide was still active (Manjunath, 1978). Denaturants such as urea (8 M) completely inactivated *A candidus* and *Rhizopus* glucoamylases when compared with *A. niger* glucoamylase which lost only 35% of activity (unpublished data). Thus, the glucoamylases from different species differ in their properties such as resistance to proteolysis and urea denaturation.

Secondary and tertiary structure

The catalytic activity of an enzyme protein is obviously related to its "active" conformation which apparently exists at the optimum pH and temperature. There is not much information in literature regarding the conformation of glucoamylases except to a limited extent in the case of glucoamylases of *A. foetidus* (Freedberg *et al.,* 1975) and *A. saitoi* (Inokuchi *et al.,* 1981,1982a, b). In our laboratory the secondary and tertiary structures of native, physically and chemically modified glucoamylases from *A. niger, A. candidus* and *Rhizopus* species were studied.

Conformation of native glucoamylases

The near ultra-violet (UV) circular dichroism (CD) spectra of glucoamylases from all the three species (*A. niger, A. candidus* and *Rhizopus* species) at the optimum pH (*i.e*., at pH 4·8) showed peaks at 289–293, 279–282,265 and 257–259 nm (figures 1, 2,3a). But glucoamylases from *A. niger* and *A. candidus* showed in addition peaks at 272–275 nm; that from *A. niger* three troughs at 285, 277 and 268 nm and that from *A. candidus* only one trough at 295 nm, whereas *Rhizopus* species enzyme had none. Thus, all the three enzymes differed in their fine structure in the near UV CD spectrum. The near UV CD spectra of two forms of glucoamylases, GM_1 and GM_2 of *A. saitoi* were significantly different (Inokuchi *et al.,* 1981). The near UV CD spectrum of GM_1 showed peaks at

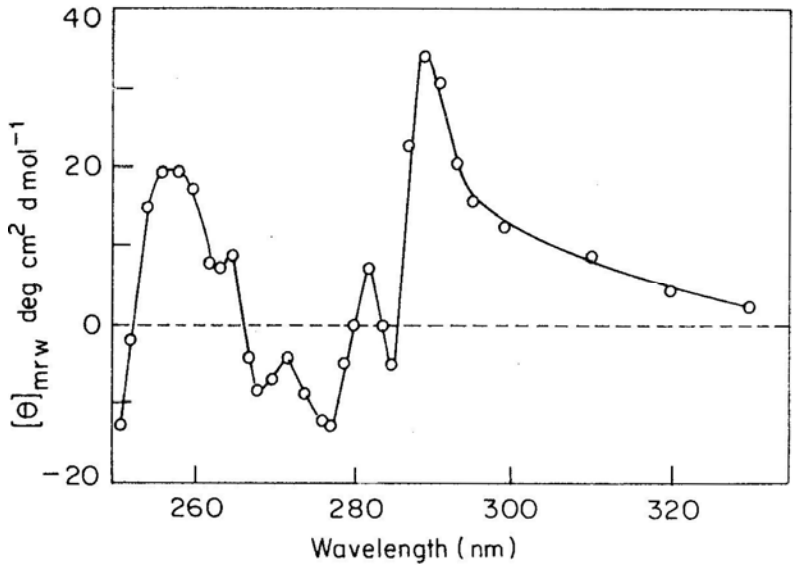

Figure 1. Near UV CD spectrum of *A. niger* glucoamylase II at pH 4·8 and 25°C.

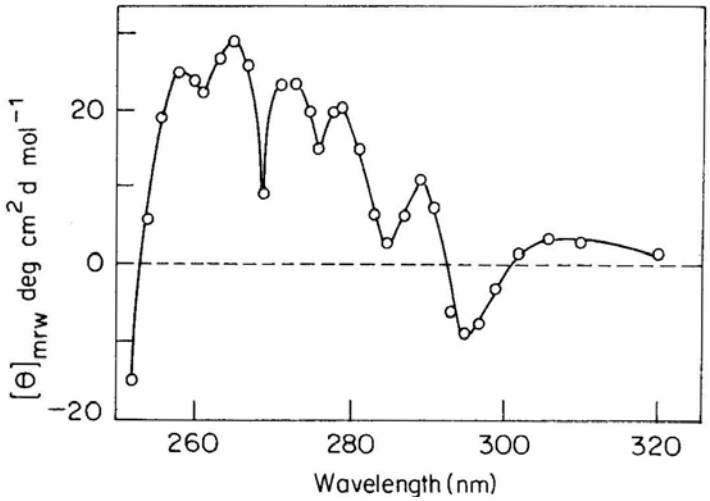

Figure 2. Near UV CD spectrum of *A. candidus* glucoamylase at pH 4·8 and 25^{0}C.

305, 290, 279, 272, 265 and 257 nm and a trough at 267 nm. Whereas the near UV CD spectrum of GM_2 showed peaks at 290 and 257 nm and troughs at 285, 277, 267 and 260 nm. In *Diazyme* glucoamylase (Freedberg *et al.,* 1975) the near UV CD spectra showed bands at 285, 278, 258 nm. The bands between 272–293 nm were probably due to tryptophan and tyrosine. Individual assignments of the bands were difficult because of the overlapping nature of tyrosine and tryptophan bands. The bands between

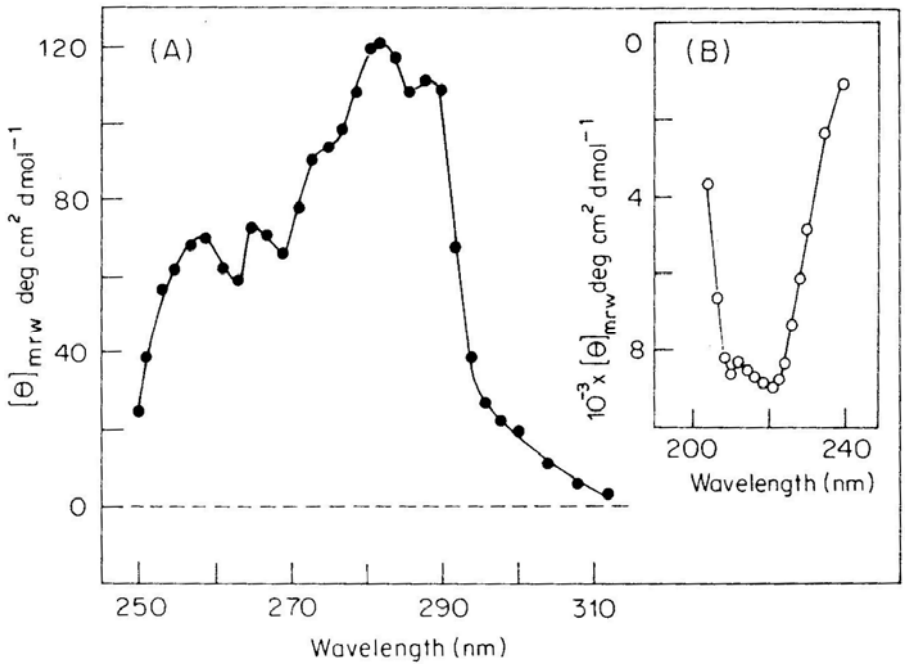

Figure 3. **A**. Near UV CD spectrum of *Rhizopus* glucoamylase at pH 4·8 and 25^0 C. **B**. Far UV CD spectrum of *Rhizopus* glucoamylase at pH 4·8 and 25^0 C.

257–267 nm were probably due to Phenylalanine and band at 304–305 nm was also possibly due to tryptophan. The overlapping contribution of cystine bands in this region cannot be ignored (Strickland, 1974). The glucoamylase from different sources do differ in their content of aromatic aminoacids and cystine content (table 1).

The far UV CD spectra of glucoamylases has negative bands at 217–220 nm and 208–210 nm differing in the magnitude and position of the bands depending upon the species. The glucoamylases from *A. niger* and *A. candidus* showed troughs at 218 and 210 nm and the glucoamylase from *Rhizopus* species exhibited trough at 220 and 210 nm. The *Diazyme* glucoamylase (Freedberg *et al.,* 1975) exhibits trough at 218 nm

Table 1. Aromatic aminoacid residues and cystine content of glucoamylases*.

	No. of residues					
Aminoacid	*A. niger* (Shenoy, 1984)	*A. candidus* (Shenoy, 1984)	*Rhizopus* species (Shenoy, 1984)	*A. niger* (Freedberg *et al.*, 1975)	*A. saitoi* GM_1 (Inokuchi *et al.*, 1981)	*A. saitoi* GM_2 (Inokuchi *et al.*, 1981)
Tryptophan	18	18	15	23	31	27
Tyrosine	19	31	42	21	30	31
Phenylalanine	28	30	33	21	23	27
Cys/2	11	11	11	10	11	10

* Calculated for 100,000 g using molecular weights of enzymes.

and 209 nm. In *A. saitoi* glucoamylases (Inokuchi *et al.,* 1981) negative troughs were seen at 217 and 208 nm in the far UV region.

The *Diazyme* glucoamylase (Freedberg *et al.,* 1975) has 15–25 % α-helix in addition to *β*-structure and disordered conformation. The infrared (IR) spectra also confirmed the data of CD by showing peaks at 1650 cm^{-1},1633 cm^{-1} and 1642 cm^{-1} characteristic of α-helix, *β*-form and unordered status respectively. The enzymes from different species contain the same amount of α-helix, but differ in their content of *β*-structure. The Greenfield and Fasman (1969) method which is based on polylysine as standard indicates a lesser amount of α-helix when compared to the method of Provencher and Glockner (1981). A comparative analysis of the far UV CD spectrum of glucoamylases from .4. *niger, A. candidus* and *Rhizopus* species by three different methods yielded data on the content of α-helix, *β*-structure and aperiodic structure is indicated in table 2. In the ORD spectrum of *Diazyme* (Freedberg *et al.,* 1975), the trough for α-helix which appears at 233 nm was shifted to 228 nm. This has been ascribed due to the presence of carbohydrate moieties in the enzyme.

Table 2. Secondary structure of glucoamylases calculated by different methods at pH 4·8.

	%		
	α	β	γ
A. niger glucoamylase			
Greenfield and Fasman (1969)	15	40	45
Provencher and Glockner (1981)	31	36	33
Chen and Yang method (1971)	18	43	39
A. candidus glucoamylase			
Greenfield and Fasman (1969)	16	33	51
Provencher and Glockner (1981)	34	24	42
Chen and Yang method (1971)	17	38	45
Rhizopus species glucoamylase			
Greenfield and Fasman (1969)	15	35	50
Provencher and Glockner (1981)	32	33	35
Chen and Yang method (1971)	15	38	47

Effect of pH: The *A. niger* glucoamylase was highly stable at pH 2·0, whereas both the *A. candidus* and *Rhizopus* glucoamylases lost their activity and conformation at this pH (table 3).

At pH 7·0, the enzyme from *A. niger* showed conformational changes; both near UV and far UV CD spectra were different from those at pH 4·8. The near UV CD spectra of glucoamylase of *A. niger* at pH 7·0 studied here were very similar to exo-glucosidase of *A. niger* reported by Freedberg *et al.* (1975).

The helical content of all the three enzymes studied here, decreased with an increase in the *β*-structure (table 3). Although, the content of ordered structure increases on storage, the activity decreases at pH 7·0.

When the pH of the solution is raised to 10·0, there is the ionisation of tyrosine

Table 3. Effect of pH on secondary structure and activity of glucoamylases.

	A. niger				*A. candidus*				*Rhizopus* species			
	%				%				%			
pH	α	β	γ	Activity	α	β	γ	Activity	α	β	γ	Activity
2·0	22	60	18	60	13	56	31	0	8	45	47	0
4·8	31	36	33	100	34	24	42	100	32	33	35	100
7·0	24	52	24	2	25	48	27	16	27	58	15	0
10·0	17	38	45	0	18	76	6	0	32	44	24	0

residues is reflected in the red shift of 257 nm band in all the three enzymes and the net charge on the protein molecule is affected resulting in the unfolding of protein molecule. At this pH, the enzyme loses its activity and structure suggesting the possible involvement of ionic linkages in maintaining the native conformation. The cystine residues are also affected at this pH and this change is reflected in the increased band intensities of 257 nm. With the cross-linking of disulfide bridges, the enzyme gets inactivated irreversibly at alkaline pH (Donovan, 1967, 1973; Donovan and White, 1971; Nozaki and Tanford, 1967). Earlier, Freedberg *et al.* (1975), Sakai *et al.* (1970) and Beychok (1965) had reported an increase in intensity of the 257 nm band and also shifting of this band towards higher wavelengths in exo-l,4-glucosidase of *A. niger* stem bromelain and lysozyme respectively. These effects may be due to the ionisation of tyrosine groups (Freedberg *et al.,* 1975; Sakai *et al.,* 1970) and also the effect upon disulfide bridges (Freedberg *et al.,* 1975; Beychok, 1965). The helical content decreased in all three enzymes studied here (table 3) and in those reported earlier (Freedberg *et al.,* 1975).

Effect of temperature: While studying the exo-l,4-glucosidase of *A. niger,* Freedberg *et al.* (1975) observed a decrease in helical content as the temperature of the enzyme solution was raised to 58°C. In our studies also, a similar phenomenon was noticed in the case of all the three enzymes studied when the temperature was raised to 60°C, thus showing the unfolding of protein molecule at higher temperatures (table 4). In the near UV CD spectrum, the changes were not so significant with an increase in temperature,

Table 4. Effect of temperature on activity and structure.

	A. niger				*A. candidus*				*Rhizopus* species			
	%				%				%			
Temperature (°C)	α	β	γ	Activity	α	β	γ	Activity	α	β	γ	Activity
15·5	20	43	37	0	32	41	27	0	30	31	39	20
25·0	31	36	33	15	34	24	42	29	32	33	35	53
30·5	28	36	36	22	27	39	34	40	23	35	42	74
60·0	25	34	41	100	19	37	44	84	19	43	38	90

i.e., the aromatic region of the spectrum is not affected by an increase in temperature. With increase in temperature, there is gradual unfolding of the molecule, and when the molecule unfolds, there may be inter- or intra-molecular cross-linking of disulfide residues resulting in aggregation of species (Freedberg *et al.,* 1975; Cassim and Yang, 1967). At higher temperatures the enzymes from *A. candidus* and *Rhizopus* species aggregate at higher protein concentrations (1 to 1·5 mg/ml). The disulfide linkages apparently play an important role in stabilizing the structure as reflected in the effect of pH and temperature on the enzyme.

Effect of urea: The glucoamylases from different sources do differ in their stability towards urea denaturation. The *A. saitoi* enzyme (Inokuchi *et al.,* 1981) did not show any changes in the CD spectra or activity in 8 M urea whereas in our studies, except in *A. niger* glucoamylase, *A. candidus* and *Rhizopus* glucoamylases lost their activity completely in 4 M urea, although unfolding of the enzyme was not complete (table 5). There was a slight loss in the secondary structure of the *A. niger* enzyme with very little changes in the near UV CD spectrum and the enzyme retained 65 % of its activity in 8 M urea. The active site of this enzyme and that of *A. saitoi* (Inokuchi *et al.,* 1981) reported earlier were perhaps not accessible to urea and thus the enzymes retained their activity and structure, whereas those from *A. candidus* and *Rhizopus* species lost their activity and structure since they were unfolded by the urea.

Table 5. Effect of denaturants on activity and structure.

	α	β	γ	
		%		Activity
A. niger				
Control	31	36	33	100
2 M urea	19	45	36	82
8 M urea	18	41	41	65
A. candidus				
Control	34	24	42	100
2 M Urea	22	43	35	60
4 M Urea	7	42	51	0
8 M Urea	4	46	50	0
***Rhizopus* species**				
Control	32	33	35	100
2 M Urea	11	65	24	40
4 M Urea	8	52	40	0

Effect of substrate: Freedberg *et al.* (1975) reported that binding of synthetic substrate *p*-nitrophenyl-α-D-glucoside resulted in slight changes only in secondary structure without any change in near UV CD spectrum. In all three cases studied here, binding of the synthetic substrate, *p*-nitrophenyl-α-D-glucoside, brought about changes in the near UV (tyrosine/tryptophan) bands and also a decrease in the secondary structure (table 6). On the basis of titration of tyrosine/tryptophan bands $[\theta]_{277}$ (figure 4) and

Table 6. Effect of substrate on structure of glucoamylases.

	%		
	α	β	γ
A. niger			
Control	31	36	33
$1 \cdot 10^{-4}$ M	27	41	32
$3 \cdot 10^{-4}$ M	26	38	37
A. candidus			
Control	34	24	42
$3 \cdot 10^{-4}$ M	18	74	8
Rhizopus species			
Control	32	33	35
$3 \cdot 10^{-4}$ M	14	42	44

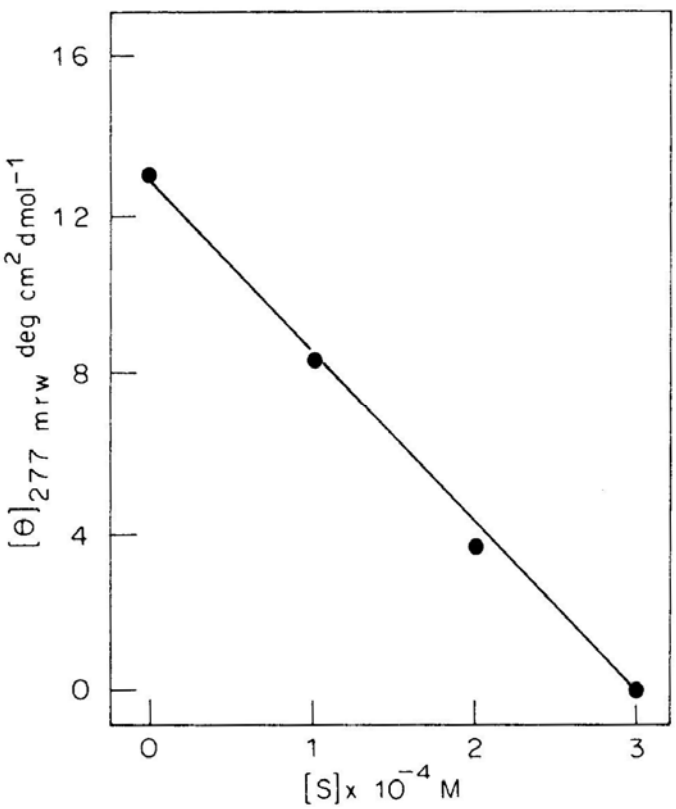

Figure 4. Effect of substrate concentration of *A. niger* glucoamylase II. The changes in $(\theta)_{277nm}$ of enzyme-substrate complex.

decrease in secondary structure, it may be concluded that substrate binds to the tyrosine/tryptophan residues and brings about a change in the conformation of the enzyme.

Carbohydrate function: All the glucoamylases so far studied are glycoproteins (Manjunath *et al.,* 1983; Ohga *et al.,* 1966; Pazur and Okada, 1967). Attempts have been made to understand the function of these carbohydrate moieties. They may be required either for activity, stability and/or for antigenecity of the enzyme. These can be checked by removing the carbohydrate residues by treating with sodium metaperiodate (Lee and Hager, 1970) or better still with anhydrous hydrogen fluoride (Manjunath and Sairam, 1982). In glucoamylase II of *A. niger* the decrease of neutral sugars by periodate oxidation was fairly rapid. In 20 min, 75 % of the carbohydrate moieties (neutral sugars) can be removed with only 20 % loss in activity (Manjunath and Raghavendra Rao, 1980). In the case of *A. candidus* and *Rhizopus* glucoamylases about 60 % and 40 % carbohydrate moieties can be removed in 20.min with 50% and 30% loss in activities respectively (Shenoy, B. C. unpublished data). The carbohydrate moieties therefore do not seem to be involved in catalytic mechanism.

Immediately after removal of carbohydrate moieties, there was a decrease in the helical content of *A. niger* glucoamylase from 31 to 27% without any appreciable changes in near UV CD bands. The enzymes still retained 80 % of activity. However, on storage at 4°C, the oxidized enzymes lost completely both activity and structure in 10 days, thus indicating that carbohydrate groups were necessary for stability of the native conformation but not for activity of the enzyme (table 7, figure 5).

Table 7. Effect of partial removal of carbohydrates on structure and activity.

	%			
	α	β	γ	Activity
Control				
pH 4·8	31	36	33	100
after 10 days	22	55	23	100
Periodate treated				
fresh	27	24	49	80
after 10 days	17	27	56	0
Heat-treated				
pH 4·8 (60°C for 20 min)	20	47	33	60
Periodate treated				
(60°C for 20 min)	3	49	47	0
Reduction with hydrogen + platinum oxide				
pH 4·8	6	44	50	142
Periodate treated	5	45	35	100

A marked decrease in heat-stability of the enzyme" from *A. niger* also occurred on oxidation. The heat-treated native enzyme lost its activity partially (40 % loss) as well as the secondary structure without any appreciable changes in near UV CD bands. On the

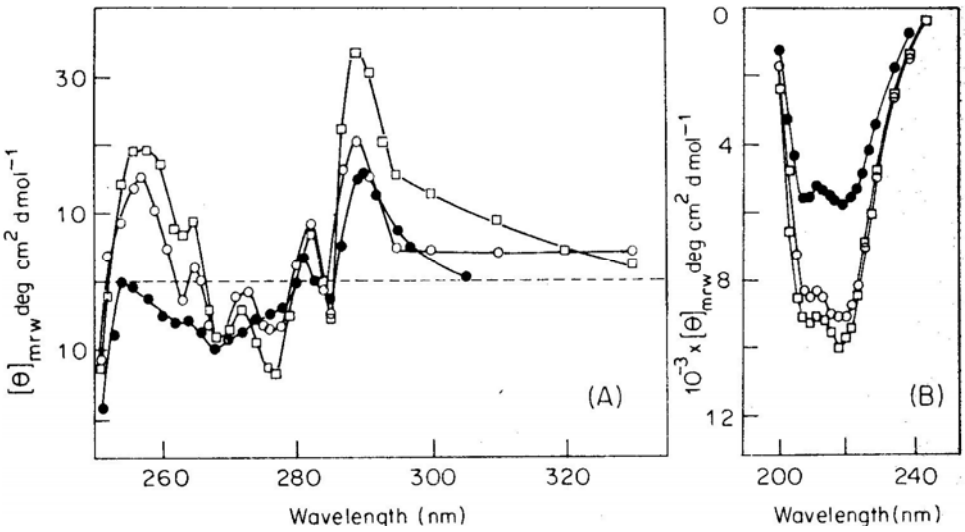

Figure 5. **A.** Near UV CD spectra of *A. niger* glucoamylase II. (□) Control; (O), periodate treated (fresh); (●), periodate treated after 10 days. **B.** Far UV CD spectra of *A. niger* glucoamylase II. (□), Control; (O), periodate treated (fresh); (●), periodate treated after 10 days.

other hand, the oxidized enzyme lost its activity and structure completely. The carbohydrate moieties thus play a role in stabilizing the enzyme against heat denaturation (figure 6, table 7).

One of the reasons for this decrease in stability of the oxidized enzyme could be due

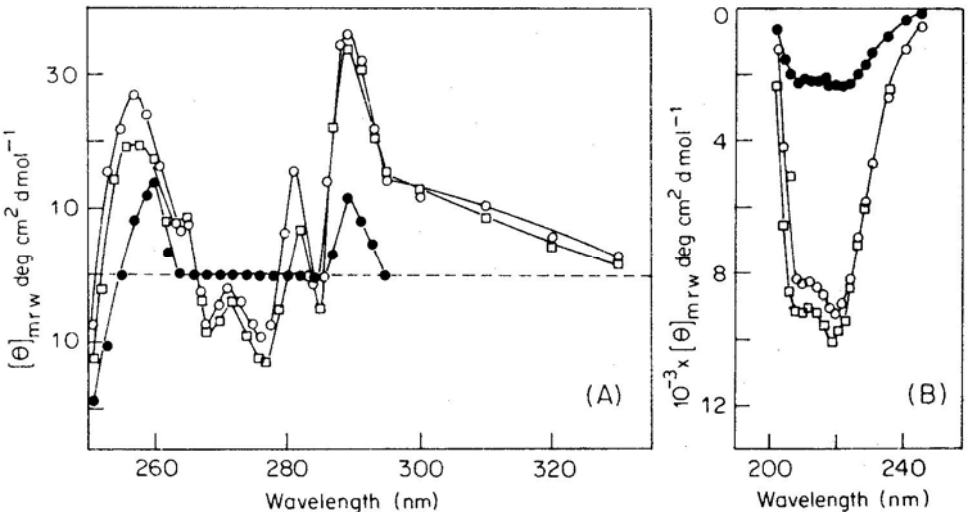

Figure 6. **A.** Near UV CD spectra of *A. niger* glucoamylase II. (□), Control; (O), heat treated; (●), periodate and heat treated. **B.** Far UV CD spectra of A *niger* glucoamylase II. (□), Control; (O), heat treated; (●), periodate and heat treated.

to the reactivity of the aldehyde moieties formed by periodate oxidation. The latter might react with the ε-amino groups of lysine or with each other in an aldol condensation resulting in loss of activity and stability of the enzyme over a period of time (10 days). Reduction of the oxidized *A. niger* enzyme by sodium borohydride resulted in a loss of activity because the pH could not be maintained at 7·0. The use of platinum oxide and hydrogen did help in regaining the activity fully and prolonging the storage life to one month. Although reduction of the enzyme prevented Schiff-base formation, it did not help in preventing the loss of activity after a month (Shenoy, B. C., unpublished data).

Reduction of the native enzyme led to a decrease not only in the helical content to 6 % but also in the band intensities of 258, 265 and 289 nm (figure 7). Unexpectedly it resulted in an increase in enzyme activity to 142%. The reduction of the periodate treated enzyme also showed an increase in activity from 80% to 100% without any appreciable changes in near UV and far UV CD spectra when compared to the reduced control. The reduced periodate treated enzyme showed a decrease in intensities of 258, 265 and 289 nm bands (figure 7) and also in helical content to 5 % when compared with the native enzyme (table 7). Thus carbohydrate groups seems to be necessary for maintaining the structural stability of the enzyme.

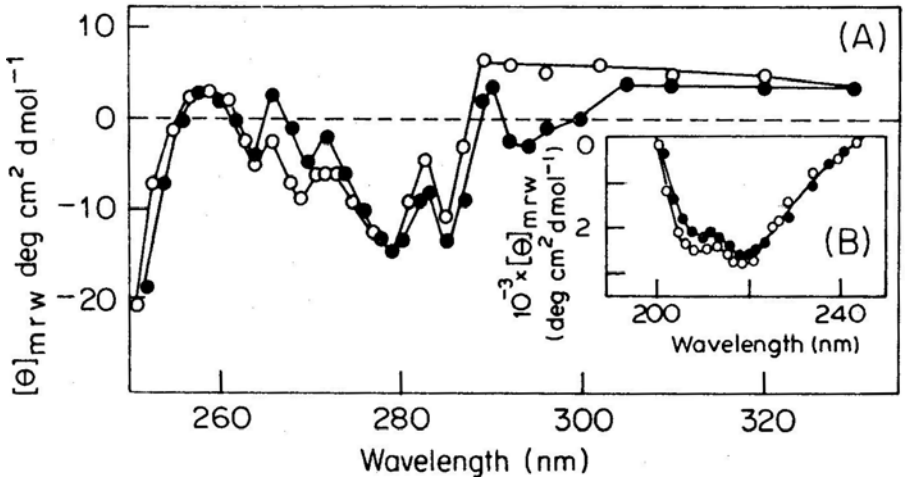

Figure 7. A. Near UV CD spectra of *A. niger* glucoamylase II. (O), Native enzyme reduced; (●), periodate treated and reduced. **B.** Far UV CD spectra of *A. niger* glucoamylase II. (O), Native enzyme reduced; (●), periodate treated and reduced.

The oxidized enzymes are however immunologically similar to the native enzymes and cross-react with the antibodies raised against native enzyme or vice versa. The carbohydrate moieties thus do not seem to be necessary for the antigenic property of the enzyme.

Chemical modification and active centre

Tryptophan groups are reported to be at the active site of glucoamylase (Jolley and Gray, 1976; Inokuchi *et al.,* 1982b; Ohnishi and Hiromi, 1976; Frankel-Conrat, 1957).

Modification of tryptophan residues by photooxidation (Barker *et al.,* 1971; Jolley and Gray, 1976; Gray and Jolley, 1973), or by N-bromosuccinimide (NBS) treatment (Inokuchi *et al.,* 1982b; Ohnishi and Hiromi, 1976; Hoschke *et al.,* 1976) resulted in loss of activity. Maltose, which is also a substrate, protects the enzyme against inactivation (Ohnishi and Hiromi, 1976). In our studies, modification of the tryptophan residues led to partial or complete loss of activity. 2-Hydroxy-5-nitrobenzyl bromide (HNBB) in the presence of urea, photooxidation at pH 9·0, NBS at pH 4·8 all abolished the activity completely. However, results of experiments using HNBB in the absence of urea, photooxidation at pH 4·8 or NBS in the presence of maltose showed that substrate protected the enzyme and two tryptophan residues seemed to be present at the active site.

The photooxidation of the enzyme suggested the presence of histidine residues at the active site (Zberebtosov *et al.,* 1976). In our studies, modification of histidine residues (0·3 and 1) resulted in only slight loss of activity (10 % and 35 %) of the glucoamylases of *A. candidus* and *Rhizopus* species respectively.

In addition, tyrosine residues were reported to be present at the active site of the enzyme, and to take part in substrate binding but not in the catalytic process(Hoschke *et al.,* 1980a, b). Modification of tyrosine either by N-acetyl imidazole or [^{125}I] resulted in partial loss of activity. Substrate maltose did not help in protecting the enzyme against modification/inactivation, Tyrosine residues may be present at the active site away from the catalytic site or they may help in maintaining the native structure of the enzymes. In the NBS-oxidized *A. saitoi* glucoamylase (Inokuchi *et al.,* 1982b), the bands at 267, 272 and 285 nm disappeared in the near UV CD spectrum with simultaneous loss in activity. This may be due to the oxidation of tryptophan and tyrosine residues.

Two to three carboxyl groups were identified at the active site of the enzyme taking part in catalytic process (Barker *et al.,* 1971; Gray and Jolley, 1973; Jolley and Gray, 1976). Modification of carboxyl groups with ^{14}C-l-cyclohexyl-3 (2-morpholinyl (4)-ethyl) carbodiimide resulted in the retention of the gross conformation of the enzyme with a complete loss of catalytic activity (Inokuchi *et al.,* 1982b). Modification of carboxyl groups resulted in a complete loss of activity of *A. candidus* and *Rhizopus* glucoamylases with modification of 10 and 28 carboxyl groups respectively. But modification in the presence of maltose showed that at least two carboxyl groups were present at the active site of the enzyme.

Only one active centre seems to be involved in breaking all the three types of α-glucosidic linkages, α-1,4, α-1,6 and α-1,3. An enzyme preparation whose carboxyl groups were modified in the presence of maltose (α-l,4-linkage) hydrolyzed both isomaltose (α-l,6-linkage) and nigerose (α-l,3-linkage) as well as maltose completely thus indicating that the glucoamylase contained only one active centre for breaking the glycosidic bonds in maltose, isomaltose and nigerose.

Binding kinetics of the subsite, active site and mechanism of action

Fungal glucoamylase hydrolyses starch, maltose and other carbohydrates having α-1,4-, α-1,6-and α-l, 3 -glucosidic linkages without any transferring action. The simplicity of its action and the availability of well-characterised substrates of low molecular weight such as maltose, enabled a study of its reaction kinetics in detail and in good accuracy.

The rate of reaction is largely dependent on the chain length of the substrate (table 8) (Ono *et al.,* 1964).

$$E + S \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} ES \xrightarrow{k_{+2}} E + P$$

$$K_m = (k_{-1} + k_{+2})/k_{+1}$$

$$V = k_{+2}[E].$$

Table 8. Kinetic parameter for various linear substrates at 15°C and pH 5· 15 (Ono *et al.,* 1964).

Substrate	Chain length (n)	K_m (M)	K_{+2} Sec^{-1}
Maltose	2	$1{\cdot}16 \times 10^{-3}$	3·02
Maltotriose	3	$2{\cdot}02 \times 10^{-4}$	12·10
Maltopentaose	5	$1{\cdot}17 \times 10^{-4}$	27·70
Maltodextrin	15	$4{\cdot}90 \times 10^{-5}$	21·00
Amylose	800	$3{\cdot}84 \times 10^{-6}$	14·40

The Michaelis constant K_m (on molar basis) markedly decrease with increasing chain length of the substrate (n-glucose units). The rate constant k_{+2} on the other hand, increases with chain length (n) upto maltopentaose but tend to decrease for larger chain lengths (table 8).

Subsite binding

As in the case of α-amylase (Iwasa *et al.,* 1974) and *β*-amylase (Kato *et al.,* 1974) which catalyze the hydrolysis of α-l,4-glucosidic linkages of starch, the action patterns have been studied quantitatively in terms of subsite affinities in relation to the binding modes and the position of the catalytic site for a series of homologous oligomeric substrates in the case of glucoamylase (Hiromi *et al.,* 1973b).

A subsite of amylase is defined as a definite part of the active site of the enzyme which specifically interacts with a glucose residue of the substrate. The affinity of a subsite towards a glucose residue, which is measured in terms of the unitary free energy decrease due to interaction, is termed subsite affinity and is denoted by A_i, where i designates the number of the relevant subsite, counting from the terminal one on which the non-reducing end glucose residue of a substrate is situated in a productive complex. According to this theory, the measurable rate parameters, the Michaelis constant K_m and the molecular binding activity K_o, can be expressed in terms of the subsite affinity and an intrinsic rate constant, $K_{int,}$ for the hydrolysis of substrate linkage in a productive complex which is assumed to be constant irrespective of the degree of polymerization (n) of the substrate and the mode of productive binding. The subsite affinity and K_{int} can be evaluated from the dependence of the rate parameters on the degree of polymerization 'n' of a series of linear substrates. Moreover, the mode of cleavage of an Oligosaccharide, *i.e.*, the probabilities of cleavage at the respective

linkages can be quantitatively predicted from the values of the subsite affinities. The apparent dependence of maximal velocity on DP arises merely from a probability of productive complex formation which is determined by the subsite affinities in the active site of enzyme (Hiromi *et al.,* 1973b; Hiromi, 1970). The subsite affinities (A_i) and the intrinsic rate constant of hydrolysis of the substrate in a productive complex (K_{int}) is given in table 9 (Tanaka *et al.,* 1983a; Sevel'ev *et al.,* 1982; Hiromi *et al.,* 1973b). The apparent zero value of the subsite A_i may be interpreted that the positive interaction affinity is compensated by the distortion free energy produced by the binding of a glucose residue at this subsite. Since glucoamylase splits off glucose residues from the non-reducing end of the substrate molecule a schematic model was proposed (figure 8).

Table 9. Values of subsite affinities A_i and the intrinsic rate constant (K_{int}) for glucoamylases of *R. delemar, R. niveus* and *A. awamori* (Tanaka *et al.,* 1983a; Hiromi *et al.,* 1973b; Sevel'ev and Firsov, 1982).

Subsite number (*i*)*	1	2	3	4	5	6	7
Subsite affinity (A_i)	A_1	A_2	A_3	A_4	A_5	A_6	A_7
Values of A_i (Kcal/mol)							
R. delemar	0	4·85	1·59	0·43	0·22	0·11	0·10
$K_{int}(S^{-1})$	77						
R. niveus	−0·48	4·96	1·36	0·54	0·32	0·23	0·07
$K_{int}(S^{-1})$	70·1						
A. awamori	−0·60	−4·50	−1·68	−0·66	−0·25	+0·66	—
$K_{int}(S^{-1})$	47·9						

* The subsites are numbered from the non-reducing end side. The catalytic site is situated between subsites 1 and 2.

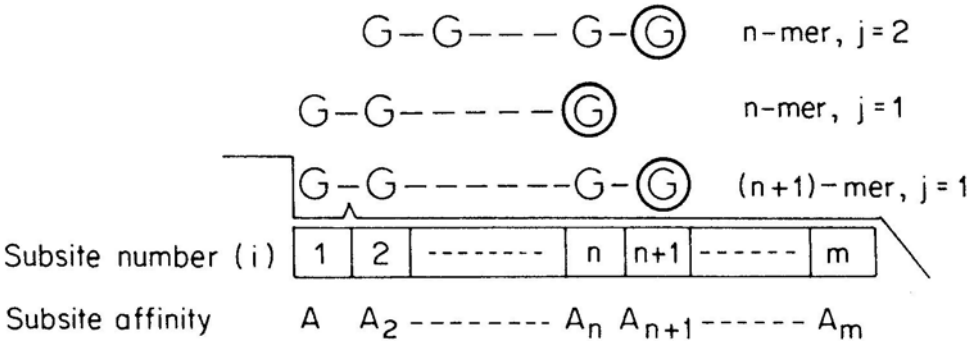

Figure 8. Schematic representation of the active site of glucoamylase and the binding modes of *n*-mer and (*n* + l)-mer substrates. G represents a glucose residue. A reducing end Ⓖ is on the right. The wedge signifies the catalytic site of the enzyme. Subsites are numbered counting from the terminal on which a glucose residue of a productive ES complex is situated; $A_i(i = 1, 2 \ldots\ldots m)$ indicates the subsite affinity (in free energy units, Kcal/mol) of the *i*th subsite. The binding modes with $j = 1$ and $j = 2$ refer to productive and non-productive complexes respectively.

On the basis of studies on the kinetic behaviour of glucoamylase and the influence of pH on the rate of reaction with the two substrates, maltose (α-1,4-) and panose (α-1,6-) and the values for ionization constants and the heats of ionization groups in the free enzyme (uncombined with the substrate), Hiromi *et al.* (1966) and Sevel'ev and Firsov (1982) concluded that a single active centre was involved in the hydrolysis of both substrates and that the essential ionizable groups 1 and 2 of the enzyme were carboxyl groups, the active forms of which were COO^- and COOH respectively.

The molecular binding affinities of α-glucose (4·1 Kcal/mol) and also other inhibitors (table 10) showed that they bind at subsite 2 of the enzyme because of their similarities to subsite affinity A_2 (Hiromi *et al.,* 1973a; Suetsugu *et al.,* 1973).

By solvent perturbation technique and difference spectrophotometry Ohnishi *et al.*

Table 10. Inhibition by analogues for glucoamylase catalyzed reactions at pH 4·5 and 25°C (Hiromi *et al.,* 1973a)

Analogue	Type of inhibition	K_i (× 10 M)	Substrate†	B (Kcal/mol)
α-D-Glucose	Competitive	0·60	1	4·1
β-D-Glucose	"	1·60	1	3·5
eq*. D-Glucose	"	1·00	1	3·8
α-D-Mannose	"	1·60	1	3·5
β-D-Mannose	"	1·50	1	3·5
eq*. D-Mannose	"	1·60	1	3·5
2-Deoxy-D-glucose	"	1·80	3	3·4
D-Glucosamine	"	0·14	3	4·9
eq*. D-Allose	—	1·70	1	3·4
α-D-Galactose	"	2·20	1	3·3
β-D-Galactose	"	2·10	1	3·3
eq*. D-Galactose	"	2·20	1	3·3
α-D-Xylose	"	2·40	1	3·3
β-D-Xylose	"	2·20	1	3·3
eq*. D-Xylose	"	2·30	1	3·3
Methyl-α-D-glucoside	Competitive	0·62	2	4·0
Methyl-β-D-glucoside	"	2·60	2	3·2
Methyl-α-D-mannoside	"	10·00	2	2·4
Methyl-α-D-galactoside	"	1·90	2	3·4
Methyl-α-D-xyloside	"	5·50	2	2·8
Methyl-α-D-glucoside	"	0·094	2	2·1
Phenyl-β-D-glucoside	"	0·32	2	4·4
Allabiose	Competitive	1·80	2	3·4
Melibiose	"	1·80	2	3·4
Sucrose	"	1·80	2	3·4
Lactose	"	36·00	2	1·6
Gentiobiose	—	1·00	2	3·8
Trehalose	—	1·60	2	3·5

* eq. = α- and β-forms in equilibrium.

† 1) *p*-nitrophenyl-α-D-glucoside.
2) phenyl 6-O-acetyl-α-D-maltoside.
3) maltose.

(1975) showed that totally there is one tryptophan and two tyrosine residues present at the subsite 1 and subsite 2 and 0·5 tryptophan and one tyrosine residues at subsite-2. So, one tryptophan and one tyrosine residues are located in subsite-1. Binding of the substrate or the inhibitor gluconolactone produces a characteristic trough at 300–302 nm in UV difference spectra which was not seen by binding of glucose, methyl-β-glucoside and cellobiose (Ohnishi *et al.,* 1975,1976). The trough was due to the effect of a negative charge produced by the ionization of a carboxyl group (present at the catalytic site) on the indole chromophore of a tryptophan residue present at subsite 1 (Ohnishi *et al.,* 1975,1976). Modification of tryptophan residues by NBS also showed that two tryptophan residues were present at the catalytic site of the enzyme and upon modification the characteristic difference spectra with a trough near 300 nm produced by maltose disappeared with simultaneous loss in the catalytic activity (Ohnishi and Hiromi, 1976).

The kinetics of binding maltose, gluconolactone and also maltooligosaccharides have been studied using stopped flow techniques by monitoring the decrease in enzyme fluorescence in relation to the subsite structure of the enzyme. The results indicate that binding consists of a two step mechanism, a fast bimolecular process followed by a slow unimolecular process (Ohnishi and Hiromi, 1978; Hiromi *et al.,* 1974; Ohnishi *et al.,* 1977; Hiromi *et al.,* 1982; Tanaka *et al.,* 1982, 1983b).

$$E + L \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EL \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} EL^{*}.$$

Where *EL* and *EL** are the enzyme-ligand complexes loosely bound and tightly bound respectively. In each case, the observed decrease in intensity of fluorescence of the enzyme protein was confirmed to be accompanied solely with slow unimolecular process. A competitive inhibitor α-D-glucose binds to subsite 2 and inhibits only the fast bimolecular process for maltose binding. On the other hand, binding of glucose inhibits the fast bimolecular process for gluconolactone, and the k_{-2} step, but not the k_{+2} step, in the unimolecular process. In the fast bimolecular process of binding, gluconolactone transiently binds to subsite 2 and maltose to subsites 2 and 3, and in the unimolecular process they relocate to subsite 1 and subsites 1 and 2 respectively accompanied by the decrease in fluorescence intensity (figure 9).

Immobilized glucoamylase

Currently, glucose production in the world is by enzymatic methods. Industries make use of bacterial α-amylase and fungal glucoamylase for the conversion of starch to glucose, which is a well established commercial process (Chibata, 1978). Due to the obvious advantages of processes using immobilized enzymes and also in order to make industrial glucose production a continuous process, several immobilization techniques such as adsorption, covalent/cross-linking, ionic binding, entrapment and making use of membrane like structures have been exploited for the immobilization of glucoamylase on various supports. The literature on immobilized glucoamylase indicates that a great deal of effort has been made to obtain a stable immobilized preparation. The various studies reported on the immobilization of glucoamylase on

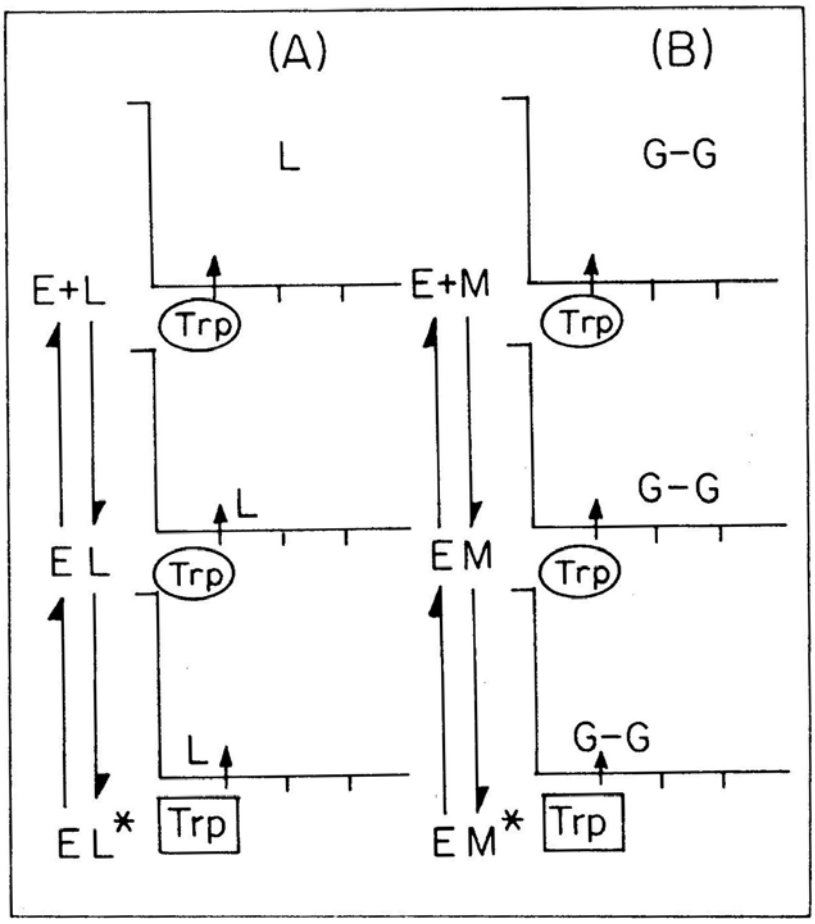

Figure 9. Proposed mechanisms of the binding processes of glucanolactone (**A**) and maltose (**B**). The wedge indicates the catalytic site. (Trp) and [Trp] indicate the emissive and the quenched states of the tryptophan fluorescence, respectively.

different supports have been extensively covered by Pitcher (1980), Chibata (1978) and Olson and Korus (1977). Of these, covalent/cross-linking and adsorption techniques seem to be extensively studied. But small scale production of glucose (3·8 kg/100 h in a reactor column) from starch using glucoamylase immobilized on 2-amino-4,6-dichloro-S-triazine-activated DEAE-cellulose and its reactor kinetics have been reported (Chibata, 1978).

The only effort on the large scale use of immobilized glucoamylase appears to be the pilot scale studies by Corning Glass Works, USA (Weetall, 1975), where glucoamylase absorbed on porous silica ceramic was used for these studies. It was calculated that 2,222 tonnes (4·6 million lbs) of glucose could be produced per year by using a 2·5 ft^3 (6 inch × 8 feet column). A pilot plant with multistage columns of 1 ft^3 volume each was operated at Iowa State University and 1000 lbs (~ 450 kg) of glucose was produced per day. During 70 days of operation, there was no detectable change of enzyme activity.

Table 11. Glucoamylase immobilized with other enzymes.

Enzymes immobilized	Method of immobilization	Support/carriers used	Use/application	Reference
Glucoamylase + glucose oxidase	Co-valent (Co-immobilized)	CNBr-activated Sepharose-4B	Model	Gestrelius *et al.*, (1972)
Glucoamylase, α-amylase and/or pullulanase	Co-valent (Separately immobilized)	DEAE-cellulose AE-cellulose (activated)	Partial hydrolysis of starch to glucose	Ger. Offfen (1976)
Glucoamylase + glucose isomerase	Co-valent (Co-immobilized)	Porous glass particles	High fructose syrup from partially hydrolysed starch	Lee (1975)
α-amylase + glucoamylase + glucose isomerase	-do-	CNBr-activated Sepharose-6MB	Single-step production of starch to high fructose syrup	Katwa and Raghavendra Rao (1983)
-do-	-do-	*Streptomyces* cells	-do-	Katwa (1984)
α-amylase + glucoamylase	-do-	Polyacrylamide gel	Hydrolysis of starch	Walton *et al.* (1973)
Glucoamylase + yeast cells	-do-	Living yeast cells	Beer wort fermentation	Hartmeier and Muecke (1982)

However, increased pressure drop in the column due to high viscosity of liquefied starch and other technical difficulties prevented its commercialization.

Recently Novo Industri (Denmark) has developed an immobilized glucoamylase preparation by cross-linking with glutaraldehyde a mixture of albumin and glucoamylase on an inert casein core, and have suggested its use for several purposes (Amotz *et al.,* 1978), but so far, none of the processes has been commercialized.

Immobilization with other enzymes

Probably due to the more beneficial effects the immobilization of sequential enzymes on the same particle/carrier or on different particles/carriers used in separate or mixed systems have also been exploited (Mosbach and Mattiason, 1976; Mattiason, 1977). Glucoamylase has been co-immobilized with glucose oxidase and studied as a model system (Gestrelius *et al.,* 1972). In most of the cases glucoamylase has been co-immobilized with α-amylase or with a mixture of α-amylase and glucose isomerase. A few example of such studies are summarized in table 11 which indicates that most of these studies have been attempted to make use of such systems in the starch industry particularly for the continuous conversion of starch to glucose or high fructose syrup. It is interesting to note (table 11) that an attempt has been made for the "single step" conversion of starch to high fructose syrup using an immobilized multienzyme system of α-amylase, glucoamylase and glucose isomerase (Katwa and Raghavendra Rao, 1983; Katwa, 1984). Glucoamylase immobilized on living yeast cells has been used for beer fermentation (Hartmeier and Muecke, 1982).

These studies indicate possibilities of using such systems. However, application of such basic concepts to industries need much more extensive investigations.

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