

The Function of the Carbohydrate Units of Three Fungal Enzymes in Their Resistance To Dehydration

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ABSTRACT

Glucose oxidase (from *Aspergillus niger*), glucoamylase (from *Rhizopus* spp.), and cellulase (from *Aspergillus niger*) of fungal origin are all glycosylated proteins. Dehydration of the three enzymes to a range of water potentials did not affect their activity. However, when more than 10% of the carbohydrate associated with the molecules was removed by periodate oxidation, the enzymes were highly susceptible to dehydration when compared with oxidized controls. Polyvinyl pyrrolidone and Dextran T500 protected the three enzymes in their oxidized state against the effects of dehydration.

The carbohydrate units of the enzymes have a function in protecting the enzymes from dehydration and this may be a contributing factor to the survival of microorganisms in environments of low water potentials. The involvement of water associated with the enzyme molecules due to the presence of carbohydrate units is considered to be the protecting mechanism.

It has been reported that some species of fungi are able to survive, and indeed attain maximum growth rates, at water potentials as low as -200 bars (20, 21). Investigations reported here were performed to determine if individual enzymes associated with fungal metabolism possess unique properties to be able to withstand dehydration. Three fungal enzymes—glucose oxidase (EC 1.1.3.4.), glucoamylase (EC 3.2.1.3.), and cellulase (EC 3.2.1.4.)—were chosen for investigation. Glucose oxidase and glucoamylase have both been shown to be glycoenzymes containing 16% and 10% to 18% carbohydrate, respectively (17). Cellulase has also been demonstrated to contain carbohydrate moieties (8, 10, 27, 28).

The function of the carbohydrate residues of glycoenzymes has not been clearly defined. However, one interesting demonstration is that carbohydrate moieties of glucoamylase stabilize the tridimensional structure of this enzyme when stored at low temperatures (19). As explanations of freezing injury of enzymes have invoked dehydration concepts (15), it was decided to examine the contribution of the carbohydrate moieties of glucose oxidase, glucoamylase, and cellulase with regard to their ability to withstand dehydration. It will be shown that partial oxidation of carbohydrate residues of these enzymes results in their increased susceptibility to dehydration.

MATERIALS AND METHODS

Enzyme Source and Assay. Glucose oxidase (EC 1.1.3.4.) from *Aspergillus niger*, glucoamylase (EC 3.2.1.3.) from

Rhizopus spp., and cellulase (EC 3.2.1.4.) from *Aspergillus niger* were purchased from the Sigma Chemical Company. Before use the three enzymes were passed through a column of Sephadex G-75 resin and the active fractions combined and concentrated using an Amicon cell with Diaflo membrane type UM 05.

Glucose oxidase was taken up in 0.1 M sodium acetate buffer (pH 5.6). The reaction mixture for the assay of enzyme activity consisted of 25 ml of acetate buffer containing 3% glucose and 100 μ l of the enzyme preparation. Activity was recorded by measuring oxygen uptake with a Beckman Model 777 oxygen analyzer.

Glucoamylase in 0.05 M sodium citrate buffer (pH 3.5) was assayed using 1% starch as substrate. The reaction was allowed to continue for 1 hr at 25 C, and activity was estimated by determining glucose released. Glucose was estimated by adding 100 μ l of glucose oxidase to the reaction mixture and by measuring oxygen uptake with the Beckman oxygen analyzer.

Cellulase in 0.05 M sodium citrate buffer (pH 3.5) was assayed using 1.5% carboxymethylcellulose as substrate. The reaction was allowed to continue at 25 C for 16 hr. Activity was measured by estimating glucose released as described above.

Periodate Oxidation of Enzymes. Periodate oxidation has been used to determine the structure of oligosaccharide units of glycoproteins (23), the role of carbohydrates associated with freezing point-depressing glycopeptides (13) and the function of carbohydrates in glycoenzymes (29). In this study, to determine the influence of the carbohydrate residues on the stability of the enzymes during dehydration, aliquots of each of the enzymes were subjected to periodate oxidation. The enzymes were exposed to 20 mM sodium periodate for different times to achieve variable carbohydrate removal. Oxidation was usually stopped by reducing excess periodate with ethylene glycol. The oxidized enzyme was then dialyzed for 24 hr against the appropriate buffer, concentrated using an Amicon cell with Diaflo membrane UM05, and passed through a column of Sephadex G-75 resin; the active fractions were used as the oxidized enzyme preparation.

Controls were subjected to the same procedure, except ethylene glycol and sodium periodate were mixed before the addition of the enzyme. The controls and treated enzymes were then assayed before and after dehydration. Residual carbohydrate content of the oxidized enzymes was determined using the method outlined by Dubois *et al.* (7).

Dehydration Procedure. The term "water potential" is used throughout this paper to describe the energy relations of the system. Water potential has been defined by Slatyer and Taylor (22) as the difference between the partial specific Gibbs free energy of water in a system compared with that of pure water.

The enzymes were dehydrated using a pressure membrane

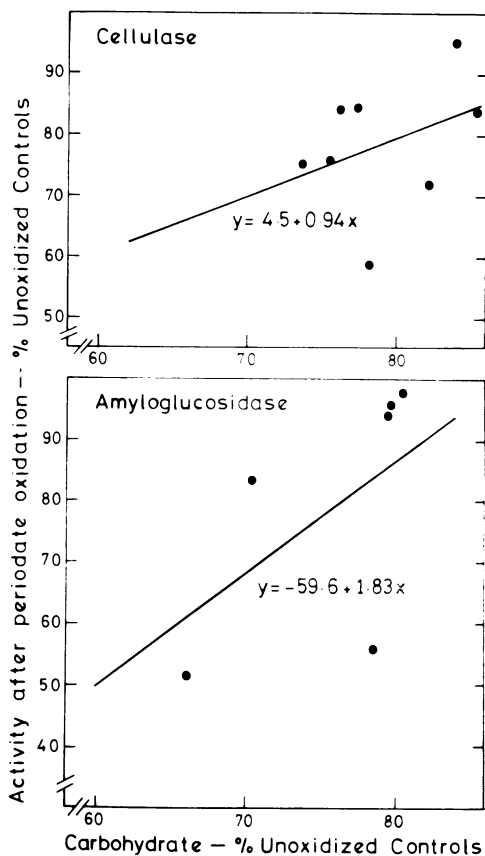


FIG. 1. Effect of periodate oxidation on the activity of glucoamylase and cellulase. The variable carbohydrate content of the molecules was achieved by different oxidation times.

apparatus (4) which allows the water potential of the enzyme system to be physically manipulated. The use of this apparatus has been described by Darbyshire and Steer (5) where they reported its use to dehydrate a number of enzymes. The apparatus consists of a cylindrical brass body closed at one end. The closed end includes a solid filter support with a spider's web pattern cut into the surface and leading to a drain. A dialysis membrane underlain by a Millipore filter is held in position over the filter support by an acrylic sleeve containing a Teflon insert which defines the effective filter area and acts as a basal seal. The filter surface has an effective diameter of 25 mm. After closing the apparatus with a screw top, pressure from a nitrogen cylinder is applied to the contents.

In the work reported in this paper, 0.4 ml of glucose oxidase and 1 ml of glucoamylase and cellulase, in their native unoxidized or oxidized forms, were added to the apparatus and a selected hydrostatic pressure was applied. This resulted in an increase in the water potential of the enzyme system and water and solutes passed through the membrane (Visking dialysis tubing 26/32, underlain by an $0.01 \mu\text{m}$ Millipore filter). Equilibration continued until the enzyme system inside the chamber was in equilibrium with the solution external to the membrane at atmospheric pressure. After this equilibrium point had been reached the applied hydrostatic pressure was released, subjecting the internal phase containing the enzyme to a tension equivalent to the applied pressure. Equilibrium time was 90 min and after pressure release the enzyme was rehydrated with 0.4 ml of acetate buffer in the case of glucose oxidase or 1 ml of citrate buffer in the case of glucoamylase and cellulase. Following rehydration, enzyme activity was assayed.

By applying different hydrostatic pressures the enzyme preparation could be dehydrated to a range of water potentials. Hydrostatic controls of the unoxidized and oxidized preparations were used as references. Here the membranes were underlain by a neoprene rubber disc, preventing equilibration, and subjecting the enzyme preparation to a hydrostatic pressure equivalent to that experienced by the treated enzyme. Hydrostatic pressure alone had no effect on enzyme activity. All operations were carried out at 25 C.

RESULTS

Effects of Periodate Oxidation on Enzyme Activity. Oxidation of glucose oxidase did not result in any change of activity of the enzyme. Only after a 2-hr oxidation time with 20 mM periodate did the activity vary from the unoxidized controls, when the carbohydrate content of the oxidized preparation was 32% of the unoxidized controls and resulted in an 11% loss of activity.

Glucoamylase and cellulase were, however, more susceptible to periodate oxidation. This effect is seen in Figure 1 where oxidation causes a loss in activity as carbohydrate content is reduced.

Effect of Dehydration on Native and Periodate Oxidized Enzymes. The three enzymes in their native state were stable to dehydration over a wide range of water potentials (Table I).

Initially the influence of carbohydrate content on the resistance of enzymes to dehydration was examined using glucose oxidase. When 70% of the carbohydrate residues were removed dehydration resulted in approximately an 80% loss of activity when compared to oxidized hydrostatic controls (Table II). The level of dehydration did not appear to influence this result. Hence the effect of dehydration to -10 bars was examined after removing variable amounts of carbohydrate from the three enzymes. Glucose oxidase (Fig. 2) and cellulase (Fig. 3) were particularly susceptible to dehydration when the carbohydrate content of these molecules was reduced by approximately 20%. Glucoamylase (Fig. 4) did not respond to dehydration as precipitously as the other enzymes, but after

Table I. Effect of Dehydration of Three Fungal Enzymes in Their Native State

Water Potential	Glucose Oxidase	Glucoamylase	Cellulase
- bars	% hydrostatic controls		
2	102.7 \pm 2.0		
4	105.3 \pm 5.5	94.0 \pm 2.8	97.4 \pm 1.0
6	100.1 \pm 4.1	105.4 \pm 1.2	93.6 \pm 1.9
8	98.5 \pm 2.3		
10		106.3 \pm 1.6	95.4 \pm 1.7
12		102.6 \pm 1.3	100.6 \pm 3.3
14		103.8 \pm 0.7	95.4 \pm 0.9

Table II. Effect of Oxidation by Sodium Periodate on Dehydration Resistance of Glucose Oxidase

The carbohydrate content of the oxidized preparation was 32% and the activity was 89% of the unoxidized control.

Water Potential	Activity after Dehydration
- bars	% hydrostatic controls
2	8.7 \pm 1.9
4	15.9 \pm 7.5
6	22.5 \pm 1.6
8	16.8 \pm 2.2

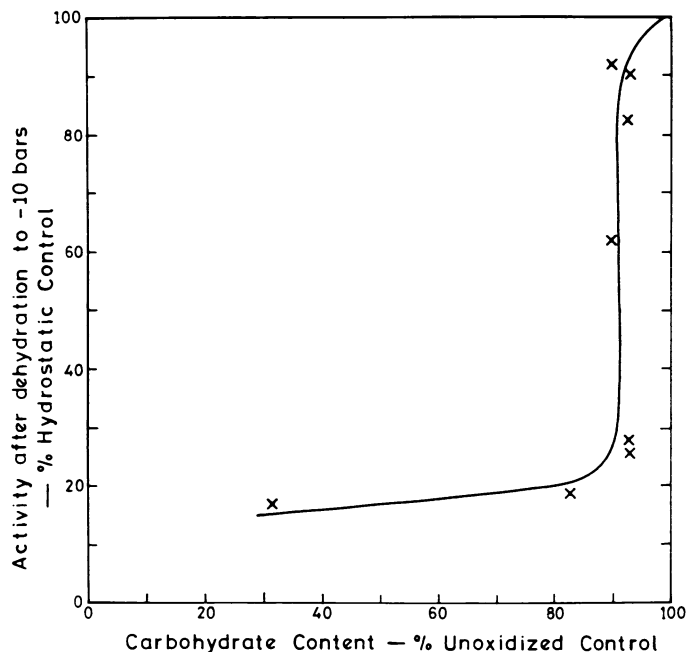


FIG. 2. Effect of dehydration to -10 bars on glucose oxidase after various amounts of carbohydrate had been removed by periodate oxidation. Controls were the oxidized enzymes subjected to a hydrostatic pressure equivalent to -10 bars.

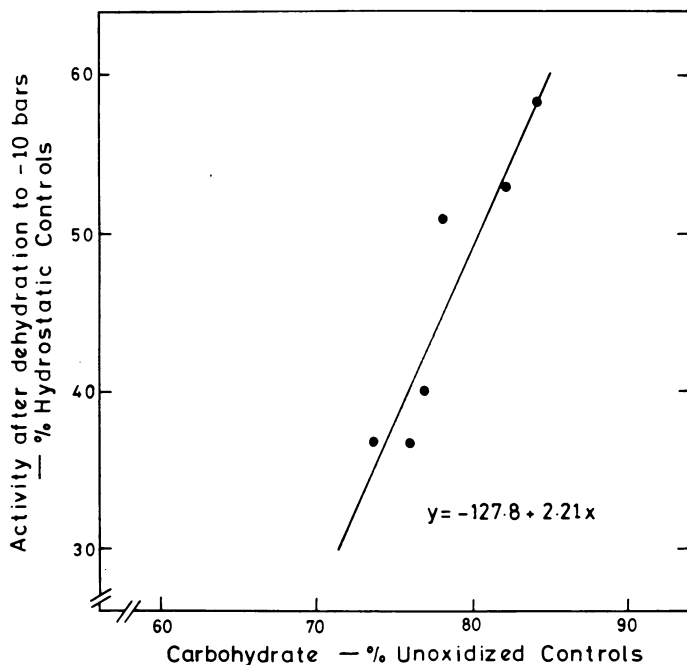


FIG. 3. Effect of dehydration on cellulase. Other details as for Fig. 2.

20% carbohydrate had been removed, dehydration resulted in a 60% loss of activity when compared to the oxidized hydrostatic controls.

Protection of Oxidized Enzymes against Dehydration. A number of compounds were tested to determine if their inclusion with glucose oxidase during dehydration could protect the oxidized molecule against such treatment. Mannose was tested because it has been shown that this sugar constitutes 13.9% of the 16% carbohydrate of glucose oxidase (17, 18).

Phosphate was included because Steer (25) has demonstrated that this ion can protect ribulosediphosphate carboxylase (EC 4.1.1.38) against the adverse effects of dehydration. The tetraalkylammonium salts and PVP have been shown to structure water (12) and were included for this reason. Furthermore, PVP and Dextran fractions (Pharmacia Fine Chemicals) are used as cryoprotectants (1) and both protect catalase (EC 1.11.1.6) against activity loss when this enzyme was dehydrated in sodium acetate buffer (6). The results in Table III indicate that the large polymers PVP and Dextran T500 protect oxidized glucose oxidase against loss of activity when these compounds are included with the enzyme during dehydration. The smaller molecules would most probably be ineffective as protectants unless they were able to bind to glucose oxidase. In the absence of binding most of these compounds would pass through the membrane during the equilibration period of dehydration.

The protection by PVP was examined more closely and the results for glucose oxidase are shown in Figure 5. Protection increases over the range 0.02% to 0.10% PVP. A concentration of 1.0% PVP completely protected cellulase and glucoamylase from the loss of activity following dehydration.

DISCUSSION

Carbohydrate associated with glycosylated proteins has been shown to contribute to the function of these molecules in a number of ways. Eylar (9) has pointed out that most extracellular proteins are glycosylated and proposed that the carbohydrate moiety acts as a label and promotes membrane transport of the synthesized glycoprotein. Removal of the carbohydrate group of glycosylated hormones abolished the ability of the hormones to interact *in vivo* with the target site (24). When terminal sialic acid residues were removed from plasma glycoprotein the desialylated proteins were rapidly removed from the blood stream and deposited in the liver (16). Glycopeptides in the blood of Antarctic fish contribute to an increase in the freezing point depression of the serum of their blood (13). These are some examples of how the carbohydrate moieties of glycoproteins contribute to their function. The results presented in this paper provide evidence suggest-

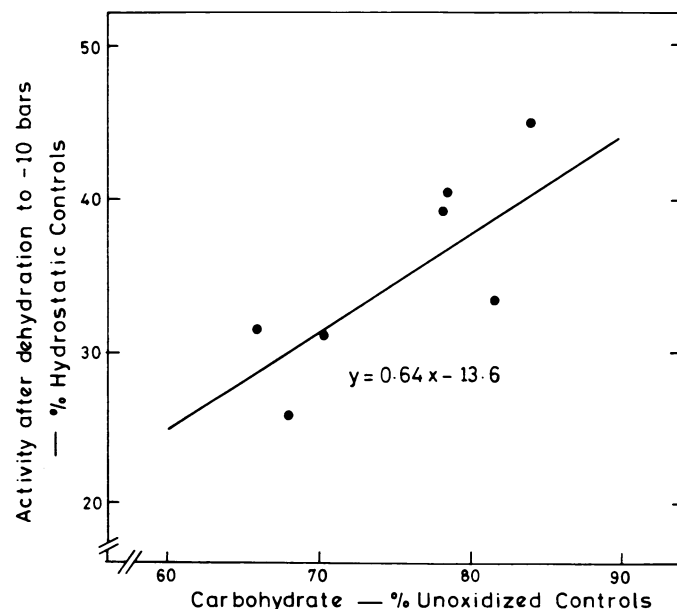


FIG. 4. Effect of dehydration on glucoamylase. Other details as for Fig. 3.

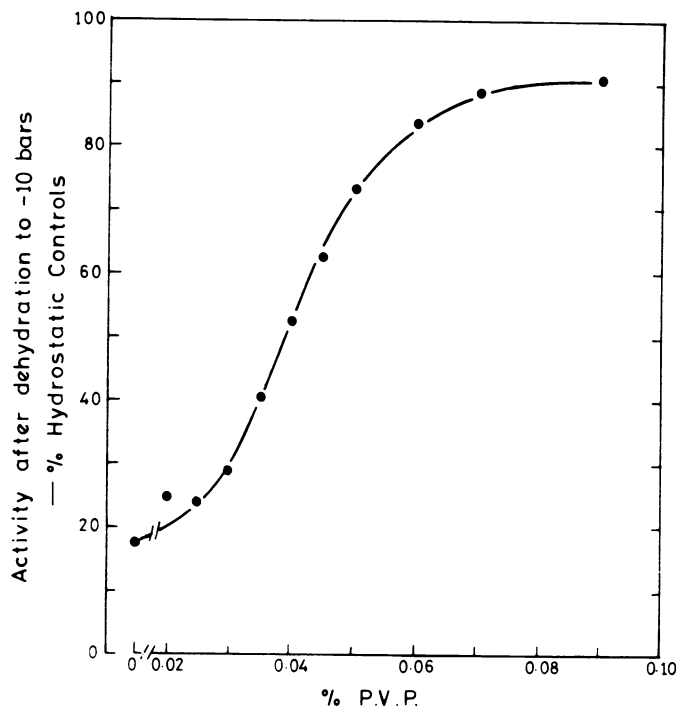


FIG. 5. Protective effect by different concentration of PVP on oxidized glucose oxidase when dehydrated to -10 bars.

Table III. Effect of Compounds on Protection of Oxidized Glucose Oxidase when Included with Enzyme and Dehydrated to -10 Bars

Oxidation conditions were 20 mM sodium periodate for 10 min. The activity was unaffected by oxidation, and the carbohydrate content after oxidation was 81% of controls. The activity of the oxidized enzyme preparation after dehydration was 22% of oxidized hydrostatic controls.

Compound Tested	Concn	Degree of Protection %
Mannose	10%	Nil
Phosphate	0.1 M	Nil
Tetrabutylammonium bromide	30%	Nil
Tetramethylammonium bromide	30%	Nil
PVP	20%	100
PVP	2%	100
Dextran T500	2%	100

ing that glycoproteins can be protected from adverse environments due to the presence of carbohydrate groups.

It is unlikely that periodate oxidation has affected the amino acid residues of the enzymes used in this study. Yasuda *et al.* (29) extensively oxidized stem bromelain with periodate and reported that this treatment did not significantly alter enzyme activity. Dextran fractions (Table III) and PVP (Fig. 5) can protect glucose oxidase and a concentration of 1.0% PVP protects glucoamylase and cellulase in their oxidized states from the effects of dehydration. This suggests that it is the carbohydrate, rather than amino acid residues, that has been altered and influences the molecules' resistance to dehydration.

Water has been shown to be associated with proteins in an ordered manner (2, 11) giving rise to descriptions such as bound and associated water. Berlin *et al.* (3) suggest that at low

humidities water is bound to specific hydrophilic sites through hydrogen bonding. As the protein swells following water absorption, increased surfaces are available for further water to be sorbed. Finally, at high humidities, the sorbed water forms an icelike or quasi-solid structure. Water is also involved in maintaining the tridimensional structure of proteins through hydrophobic bonding (26).

In the present study the results have been interpreted on the basis of water associated with the enzymes. In their native state it is suggested that carbohydrate moieties form important hydrophilic sites to which water molecules are bonded. When the enzymes are dehydrated in their native states the integrity of the molecules is retained by bound water preventing the formation of unfavorable conformations. However, upon removal of carbohydrate, water is no longer able to bind to the molecules to the same extent and they are unable to retain their native conformations when dehydrated. Alternatively, new associations upon rehydration may result in random distribution of hydrophobic complexes reducing the enzymes' activity (14).

The protection of the oxidized molecules by PVP against dehydration is difficult to explain. In cryobiological systems, protection by PVP has been attributed to the PVP causing an increase in the vapor pressure of the ice around a biological entity as compared to the system in the absence of PVP. Thus, in the presence of PVP, the vapor pressure gradient is less and dehydration is reduced (15). In the situation described in this paper, the presence of PVP would ensure larger amounts of water in the system. It is possible that water bound to PVP may be shared by the enzyme molecules and replace the need for its hydrophilic carbohydrate surfaces. Alternatively, water associated with PVP may prevent dissociation of hydrophobic groups or, if new hydrophobic associations are made, the presence of the PVP may enable the association to occur in a less random manner that is more favorable for the maintenance of activity.

It remains to be determined if specific sugars contribute to the stability of these enzymes to dehydration. However, the results presented in this paper have physiological implications connected with fungal ecology. As mentioned earlier (20, 21), some species of fungi are able to survive, and attain maximum growth rates at water potentials as low as -200 bars. A contributing factor to their survival must be the protection of extra-cellular enzymes afforded by the presence of associated carbohydrate moieties.

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