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Immunochemical relationship between glucoamylases I and II of *Aspergillus niger*

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Abstract. Rabbit antisera were prepared against the purified glucoamylases I and II of *Aspergillus niger*. Relationships between the two enzyme forms were investigated by using the antisera in immunodiffusion and immunoinhibition experiments. Both the forms of glucoamylase gave a single continuous precipitin band demonstrating very close structural resemblance. They gave almost identical immunoprecipitation patterns and had the same equivalence points indicating that the two forms of *A. niger* gluoamylases were immunologically identical. The enzyme treated with periodate was immunologically identical with the controls and had slightly less enzyme activity but showed greatly reduced stability on storage at 4° C.

Keywords. Glucoamylases; immunochemical relationship; *Aspergillus niger*.

Introduction

Two forms of glucoamylases (I and II) (EC 3.2.1.3) occur in *Aspergillus niger* and their properties have been described (Pazur and Ando, 1959; Pazur and Kleppe, 1962; Fleming and Stone, 1965; Lineback *et al*., 1969; Pazur *et al*., 1971; Venkatramu *et al*., 1975; Manjunath and Raghavendra Rao, 1979). These enzymes possess many similar properties such as the pH and temperature optima, action pattern on oligosaccharides and ability to cleave ∝ -1,4; ∝ -1,6; and ∝-1,3 bonds between glucose moieties, amino terminal amino acid, glycoprotein nature etc. On the other hand, detailed examination of the literature shows many disparate properties of glucoamylases I and II, such as molecular weight, electrophoretic mobility, amino acid content and carbohydrate content. However, none of these properties by itself can differentiate these two enzymes. Immunological tests can apparently distinguish related proteins (Aw and Hobbs, 1968; Dingle *et al*., 1971; Carroll and Robinson, 1972; Srivastava and Bentler, 1972; Matsuzaki *et al*., 1974; Messer and Dean, 1975; Phillips *et al*., 1975; Hayashi and Nakamura, 1976). It was therefore of interest to study the immunochemical behaviour of these two enzymes to elucidate structural relationships. In this paper, we report the results of such a study using the techniques of immuno-double diffusion and immunoprecipitation.

Materials and Methods

The materials used in the study were procured from the following sources: Glucozyme (glucoamylases from *A. niger*) from Anil Starch Products, Ahmedabad; Noble Agar

and Freunds's Complete Adjuvant from the Difco Laboratories, Detroit, MI, USA; soluble starch from the British Drug House, Bombay; glucose oxidase and peroxidase from Sigma Chemical Company, St. Louis, MO, USA; o-dianisidine from the CSIR Centre for Biochemicals, V.P. Chest Institute, Delhi. All other reagents used were of analytical grade unless otherwise stated.

Homogenous preparations of glucoamylases I and II were obtained according to the procedure described previously (Manjunath and Raghavendra Rao, 1979).

Glucoamylase activity was assayed as described earlier (Manjunath and Raghavendra Rao, 1979) using soluble starch as substrate. Protein was estimated according to the procedure of Lowry *et al*. (1951) using bovine serum albumin as the standard. One unit of glucoamylase releases one micromol of glucose per min under assay conditions.

Preparation of antisera. The rabbits were bled before immunization to provide control sera. The glucoamylase solution containing 10 mg of protein in saline was emulsified with an equal volume (5 ml) of complete Freund's Adjuvant. Rabbits were given intracutaneous or intramuscular injections of the antigen (1 ml) at multiple sites at 10-day intervals over a period of 50 days, and were bled from the marginal ear vein 15 days after the last injection. The blood was allowed to clot at 25°C for 2 h and the serum was collected by centrifugation at 3000 *g* for 10 min. Antisera A and B were derived from rabbits which received homogenous preparation of glucoamylases I and II isolated from Glucozyme, respectively. Antiserum C was derived from a rabbit which received injections of a dialysed aqueous extract of Glucozyme. Control sera and antisera were stored at –20° in presence of 0.2% sodium azide as preservative.

Immuno-double diffusion analysis by the method of Ouchterlony (1967) was performed at 4°C by using 20 μ 1 capacity wells in 1.5% agar in 20 mM KH_2PO_4–Na_2HPO_4 buffer, pH 7.6 containing 0.15 M NaCl and 0.01 % sodium azide. After 16-20 h of diffusion, white precipitin bands became visible and plates were photographed under dark ground illumination (Ouchterlony and Nilsson, 1973).

Immunoelectrophoresis was performed at pH 8.6 as described by Dean (1974). Immunoelectrophoresis plates (8.4 × 10 cm) were coated with 12 ml of 1.5% agar in 0.05 M Veronal buffer pH 8.6 containing 0.01% sodium azide. The antigen well had a capacity of 5 μ 1 and the antiserum trough of 80 μ 1. Electrophoresis was carried out for 6 h at 300V, 10 mA and immunodiffusion was carried out at 4°C for 2 days to permit complete formation of precipitin lines. The plates were photographed under dark ground illumination (Ouchterlony and Nilsson, 1973).

Immunoprecipitation. A constant amount (100 μ 1) of suitably diluted enzyme (0.1 mg/ml) was placed in a series of test tubes (10 × 0.5 cm). Then antiserum (0-100 μ 1) was added to the tubes and the volume made upto 500 μ 1 with 20 mM phosphate buffered (0.15 M) saline pH 7.0. After incubation at 37°C for 2 h, the tubes were stored at 4°C for 36 h (control experiments for various time-intervals showed that incubation was maximum after 36 h) and centrifuged at 1500 *g* for 15 min. The supernatant was removed and 100 μ 1 assayed for glucoamylase activity by the normal procedure. The equivalent points were calculated by plotting the activity remaining in the supernatant against the volume of the antisera in the incubation mixture.

A control experiment showed that the antisera had no glucoamylase activity. In further control experiments with normal rabbit serum in place of antiserum, no immunoinhibition of glucoamylase activity was observed.

Periodate treatment. Samples of enzyme (4 mg/ml) were separately treated in the dark with 0.1M $NaIO_4$ in 0.05 M sodium acetate buffer (pH 4.5) at 25°C for 20 min, 1 h and 2 h. The reaction was stopped by the addition of a 5-molar excess of thioglycollate and the reaction mixture passed through a Biogel-P 6 (20 × 0.5 cm) column equilibrated with 0.05 M sodium acetate buffer pH 4.8. The enzyme was eluted by the same buffer. It was then stored at 4°C and the activity periodically determined.

Results

Glucoamylases I and II of Glucozyme (*A niger* glucoamylase) were purified by the method described elsewhere (Manjunath and Raghavendra Rao, 1979). The final, freeze-dried products, glucoamylases I and II were found to be homogeneous by ultra centrifugation and by polyacrylamide gel electrophoresis methods and had specific activities of 86 and 91 units respectively.

Immunoelectrophoresis and purity. The purity of the glucoamylases was also tested by Immunoelectrophoresis against polyspecific antiserum C (raised against dialyzed queous extract of Glucozyme) and antisera A and B. Antiserum C was found to precipitate at least five proteins in the crude Glucozyme extract. With antisera A and B, a single precipitin line was observed on Immunoelectrophoresis of the homogenous enzymes (figure 1). Thus by immunoelectrophoresis also, glucoamylases I and II were found to be homogeneous.

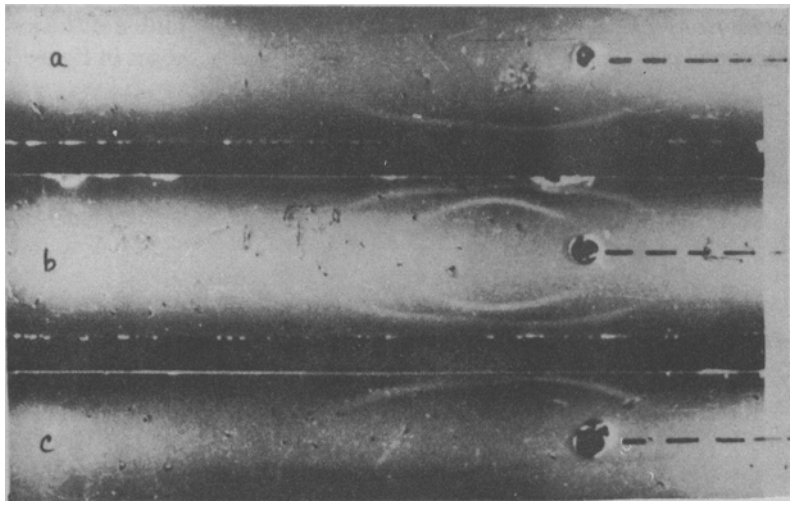

Figure 1. Immunoelectrophoresis of purified preparations of glucoamylases I (a) and II (c) and crude glucozyme extract (b) using polyspecific antiserum C (raised against crude-glucozyme extract).

Immunoprecipitation. The immunoprecipitation-inhibition patterns are shown in figure 2. The precipitation curves for glucoamylases I and II with antiserum A

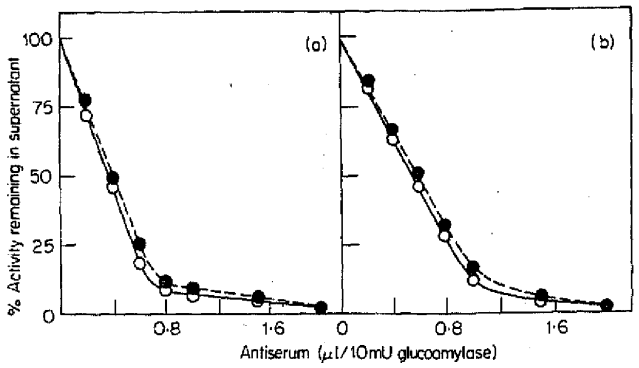

Figure 2. Immunoprecipitation curves of glucoamylases I and II with antiserum A (a) and antiserum B (b). Glucoamylese I, ●; Glucoamylase II, O.
Experimental details of assay procedure are described in materials and methods.

were identical (figure 2a). The equivalence points for glucoamylases I and II, were 71 μl and 69 μl antiserum/unit of enzyme activity respectively. Precipitation curves obtained with antiserum B were also similar (figure 2b). The equivalence points with this antiserum for glucoamylases I and II were 109 μ1 and 108 μ1 of antiserum/unit of enzyme activity, respectively.

The supernatant obtained by centrifugation (10,000 *g*; 10 min) of a reaction mixture containing glucoamylase I and its equivalent or slightly less of antiserum A does not inhibit glucoamylase II. In converse experiments, no inhibition of glucoamylase I was noticed. Hence the antisera A and B did not appear to contain antibodies specific only for either glucoamylase I or glucoamylase II.

Ouchterlony immuno-double diffusion. The immuno-double diffusion patterns of glucoamylases I and II against antisera A and B are shown in figure 3. In both

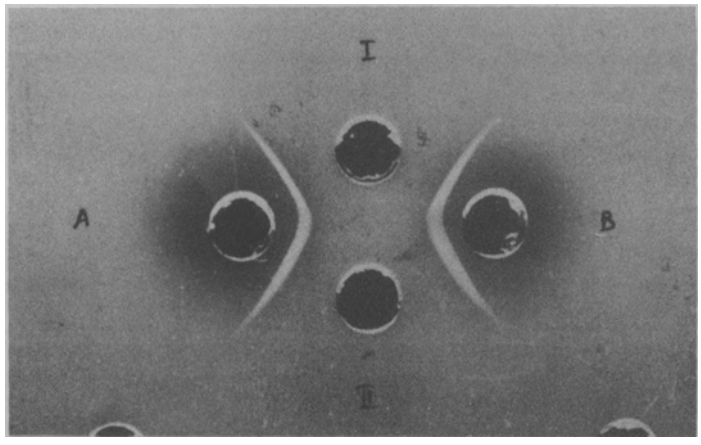

Figure 3. Ouchterlony immuno-double diffusion of glucoamylases I and II.
A, Antiserum A; B, Antiserum B.

instances, only one sharp precipitin line was obtained which was continuous in adjacent diffusions of glucoamylases I and II; there was no evidence of spur formation.

Effect of treatment with periodate . Whether the carbohydrate moiety was essential for antigenic and enzyme activity and for stability was checked by periodate treatment. The results are shown in figure 4 (a, b and c). Even when 75% of the carbohydrates of the glycoprotein enzyme were stripped, enzyme activity and antigenic behaviour were not affected, but the storage stability at 4°C was greatly reduced.

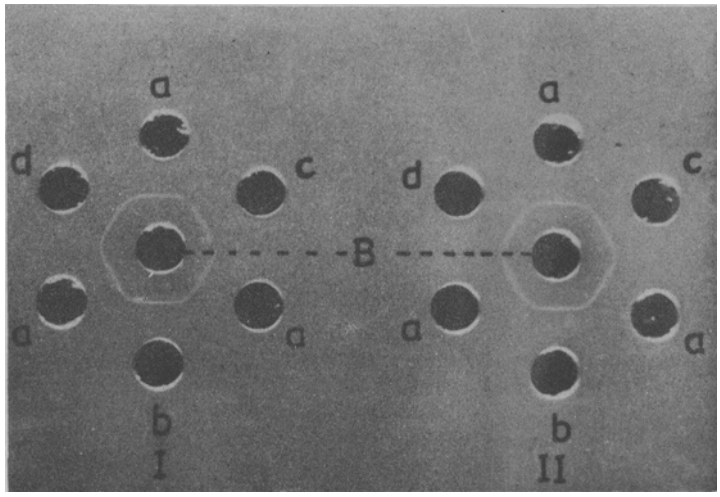

Figure 4. Immunological behaviour of the periodate-treated glucoamylases I and II.
(**a**) Immuno-double diffusion pattern of a, native enzyme and b, c and d, enzyme treated with periodate for 20, 40 and 60 min respectively. B – – – – antiserum for glucoamylase II.

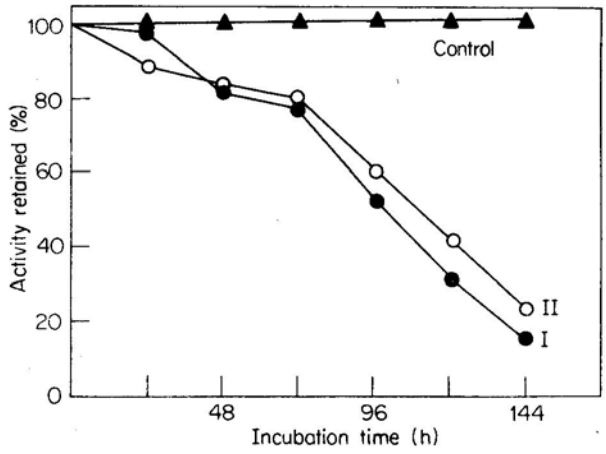

Figure 4. (**b**). Activity (O) and carbohydrate (●) content of glucoamylase after treatment with periodate.

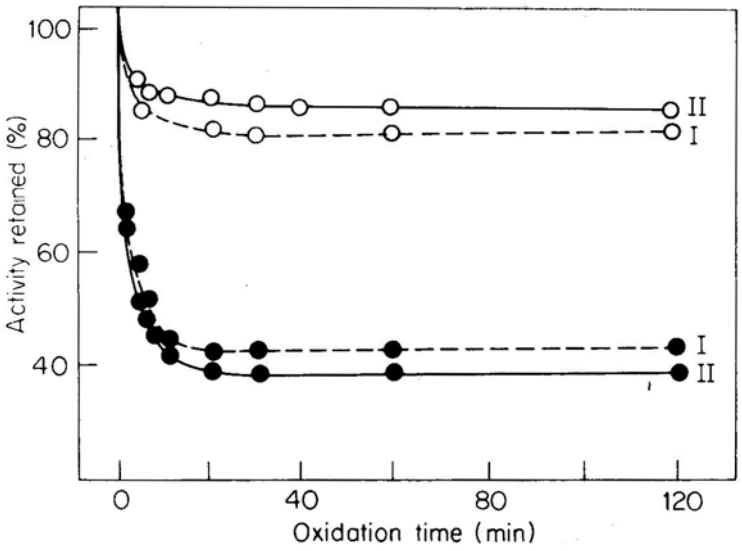

Figure 4. (c). Storage stability of glucoamylases I and II treated with periodate for 20 min. Aliquot samples of the enzyme stored at 4°C were withdrawn at time periods indicated in the figure and assayed for activity. An enzyme sample not treated with periodate but stored at 4°C served as the control. Activity of the control was normalized to 100 and per cent activity retained was plotted against time of storage.

Discussion

Immunoprecipitation has been shown to be a very sensitive technique to study the immunological relationships of closely related forms of enzymes. Examples are human liver hexosaminidases A and B (Carroll and Robinson, 1972; Srivastava and Bentler, 1972), chicken liver cathepsin D isoenzymes (Dingle *et al*., 1971), human liver ∝ mannosidase A and B (Phillips *et al*., 1975), bacterial amylases (Matsuzaki *et al*., 1974), human amylases (Aw and Hobbs, 1968), rat ∝-amylases (Messer and Dean, 1975) and fungal glucose oxidase (Hayashi and Nakamura, 1976). In the present investigation, this technique as well as immunodiffusion were used to find out the immunological identity/disparateness of the two forms of *A. niger* gluco-amylases.

Precipitation of purified glucoamylase I by antiserum B and precipitation of purified glucoamylase II by antiserum A showed that the two forms of glucoamylases were very closely related. Further, the similarity of their equivalence points and the lack of spur formation (figures 3 and 4a) in the continuous precipitin line obtained when purified glucoamylases I and II were placed in adjacent wells in an immuno-diffusion experiment suggest that they are immunologically identical. The absence in the antiserum of antibodies very specific for either glucoamylase I or II supports this conclusion. The differences noticed in the immunological equivalents (figure 2) are perhaps due to the differences in the concentration of the antibodies in antisera A and B. But the equivalents seem identical for both forms of the enzyme with either antiserum A or B. Carbohydrates are known to be antigenic, e.g., capsular

polysaccharides of *Pneumococci*. But in the present instance, the carbohydrate moiety of the glucoamylases do not seem to have a role in the antigenicity of the enzyme, since the removal of as much as 75% of the carbohydrate had no effect on the immunological behaviour or significantly on the activity of the enzyme: however, categoric Statements regarding this cannot be made yet since the periodate treated enzyme still contained much carbohydrate (25% of the original). As other experiments have indicated, the carbohydrate moiety seems to stabilize the enzyme as seen in the studies on the storage of periodate treated enzyme.

Glucoamylases I and II of Glucozyme, which are easily separable from one another, differ in a number of physical and chemical properties (Manjunath and Raghavendra Rao, 1979). Yet the fact that they are immunologically "identical" according to the tests conducted, is unexpected. The possibility that the two forms might arise as artifacts due to proteolysis or deamidation has been considered. But this has been deemed very improbable because of many facts such as the absence of proteolytic activity in the crude enzyme powders, the mild procedural conditions during the preparation of the enzymes, very similar molecular weights and specific activities but fairly significant differences in composition with respect to carbohydrate and amino acids and each form of enzyme being present in considerable proportion (I:II 3:7). Therefore the results in the present investigation indicate that there is a close structural and functional relationship between the two forms of glucoamylases in *A. niger* (Glucozyme).

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