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Structure and Properties of a *Cephalosporium acremonium* α-Galactosidase

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The amino acid and sugar composition of the enzyme protein, the effect of urea, sodium dodecyl sulphate and Concanavalin A on the purified α-galactosidase (EC 3.2.1.22) from the mold *Cephalosporium acremonium* has been studied. The results obtained by gas liquid chromatography indicated the presence of *N*-acetylglucosamine, mannose, galactose and *N*-acetylneuramic acid in the molar proportions 2:7:3:11. The presence of two types of Asn-linked oligosaccharide structures in the enzyme molecule is assumed. The α-galactosidase liberates α(1-3), α(1-4) and α(1-6)-linked D-galactose units from various synthetic and natural substrates which have been tested. The effects of pH, substrate concentration and temperature on the catalytic activity of the enzyme are described. The purified α-galactosidase also exhibited a lectin activity with an affinity towards glucose, and to some extent mannose.

α-Galactosidase, an enzyme widely distributed in nature and important in the metabolism of various galactose-containing compounds in the animal and plant kingdoms, has attracted increasing attention over the last two decades [1-3]. α-Galactosidase (α-D-galactoside galactohydrolase, EC 3.2.1.22) which catalyzes the hydrolysis of the α-galactosyl moiety have great potential applications (biotechnology, food and sugar industry, medicine). The enzyme is also useful for structural analysis and for elucidation of the biological functions of complex natural compounds, since α-linked galactosyl units are constituents of many oligosaccharides, polysaccharides, glycoproteins and glycolipids. Few examples of enzyme possessing lectin (hemagglutinin) activity have been reported but some α-galactosidases from higher plants have been shown to be lectins [1]. It has been noted that the α-galactosidase from *Vicia faba* is a unique example of an enzyme that displays true lectin

Abbreviations: p-NPG, *p*-nitrophenyl-α-D-galactopyranoside; 4-MUG, 4-methylumbelliferyl-α-D-galactopyranoside; HU, hemagglutinin unit: PBS, phosphate buffered saline; SDS, sodium dodecyl sulphate; ConA, Concanavalin A; WGA, wheat germ agglutinin; LCA, *Lens culinaris* agglutinin; PHA, phytohemagglutinin from *Phaseolus vulgaris*.

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activity, showing affinity towards α-galactosides for its catalytic activity and towards glucose/mannose for its lectin activity [4]. Compared with the α-galactosidase of higher plants, the investigations of biological activities of the enzyme from microorganisms have been neglected. The specificity of the microbial enzyme was studied by using chiefly such simple natural substrates as raffinose or melibiose and rarely by using complex glycoproteins or glycolipids.

Our previous paper dealt mainly with the isolation and purification of α-galactosidase from the mold *Cephalosporium acremonium*, some of the physicochemical properties (molecular weight, 240,000; pI, 4.96; lectin chromatography on ConA, WGA,PHA and LCA) of the enzyme, demonstrated to be homogeneous by disc gel electrophoresis, were also described [5, 6]. This study reports the general and structural properties of the purified enzymes of special interest are the experiments demonstrating the glycoprotein nature of α-galactosidase and its lectin properties. The specificity of *C. acremonium* α-galactosidase studied extensively by using various glycosides, oligo- and polysaccharides, glycoprotein and glycolipid is also discussed.

Materials and Methods

Microorganism and Cultivation

The mold *Cephalosporium acremonium* was chosen among more than 250 various cultures of yeasts, actinomycetes and molds tested for their α-galactosidase activity. Also the induction medium for higher enzyme production was used (g/l): K_2HPO_4, 2.0; $(NH_4)_2SO_4$, 5.0; $MgSO_4.7H_2O$, 0.3; $CaCl_2$, 0.3; raffinose, 0.15; yeast extract, 0.05; soybean flour, 20.0 [5]. Cultivation was continued for 72 h and the mycelia were collected by centrifugation.

Chemicals

p-Nitrophenyl-α-D-galactopyranoside and other *p*-nitrophenyl glycosides were purchased from Sigma (St. Louis, MO, USA), 4-methylumbelliferyl-α-D-galactopyranoside and melibiose were from Koch-Light Laboratories (Colnbrook, UK) and raffinose from VEB Berlin Chemie (Berlin, DDR). We are grateful to the following persons for their generous gifts: to Dr. V. D. Scherbukhin (USSR) for galactomannan from *Lagonichium farctum* [7], to Drs. E. M. Baskaeva and H. J. Wiedershein (USSR) for ceramide trihexoside [8], to Dr. L. M. Likhosherstov (USSR) for blood group B substance [9] and to Dr. A. I. Usow (USSR) for Galα(1-3)Gal.

All other chemicals were obtained from commercial sources and were of the highest grade available.

α-Galactosidase Purification

If not otherwise stated all procedures were carried out at 4°C, with the exception of enzyme incubation which were performed at 37°C. The culture supernatant after fractionation with ammonium sulfate (60-90%) and desalting by ultrafiltration with an Amicon XM-100 mem-

brane was applied to a column of DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Sweden]. The column was eluted with a linear gradient of NaCl (0.3 - 0.8 M), fractions rich in the α-galactosidase were combined and after ultrafiltration the enzyme solution was applied to a column of hydroxyapatite eluted with a sodium phosphate buffer, with a linear concentration gradient of 0.02 - 0.7 M and a linear pH gradient (7.0 - 5.1). After recycling through the hydroxyapatite column homogeneous α-galactosidase (demonstrated by disc gel electrophoresis) was obtained [5].

Enzyme Assays

α-Galactosidase activity was routinely measured with p-NPG by the procedure described previously [5]. The assay mixture contained 0.3 ml of 0.1 M sodium citrate buffer pH 5.5; 0.1 ml of enzyme solution and 0.1 ml of 1.0 mM p-NPG. After incubation for 10 min at 37°C the reaction was terminated by the addition of 2 ml of 1 M Na_2CO_3. The quantity of *p*-nitrophenol liberated was measured at 405 nm.

Degradation of 4-MUG. The reaction mixture contained 5 µl of enzyme solution, 20 µl of 2.5 mM substrate solution in 0.1 M sodium citrate buffer pH 5.5 and was incubated at 37°C for 10 min. The reaction was terminated by the addition of 4 ml of 0.4 M glycine buffer pH 10.5. Liberated 4-methylumbelliferone was measured fluorimetrically using a BIAN 1300 fluorimeter with excitation filter 365 nm) and emission filter (435 nm).

Degradation of Melibiose and Raffinose. In the case of melibiose, the reaction mixture contained 0.2 ml of enzyme solution; 0.2 ml of 0.1 M sodium citrate buffer pH 5.5; 0.2 ml of 0.01 M substrate solution and 0.4 ml of distilled water. After incubation at 37°C for 10 min, the quantity of D-glucose liberated was determined by the glucose oxidase method in the modification of Scherbukhin *et al.* [10]. When the raffinose was used as substrate the assay mixture contained 0.25 ml of 0.06 M raffinose solution; 0.25 ml of 0.1 M sodium citrate buffer pH 5.5 and 0.5 ml of enzyme solution. After incubation for 10 min at 37°C, 1.0 ml of Somogyi reagent was added to stop the reaction and the quantity of D-galactose liberated was determined by the Nelson Somogyi procedure [11].

Degradation of Ceramide Trihexoside. Ceramide trihexoside (75 µg in 0.1 ml of 0.05 M sodium citrate buffer pH 5.1 containing 0.2 M of sodium taurodeoxycholate) was incubated with α-galactosidase at 37°C for 24 h. The reaction was stopped by the addition of four volumes of butanol and the remained galactose was detected by liquid chromatography using the carbohydrate analyser LC-200 (Biotronic, West Germany)

Degradation of Blood Group B Glycoprotein. A stock solution of 25 mg of B glycoprotein in 0.7 ml of 0.02 M sodium phosphate buffer pH 6.05 was prepared, 250 µl of this solution was incubated with 300 µl of the enzyme solution in the same buffer at 37°C for 3 h. The free galactose released was detected by liquid chromatography using carbohydrate analyser mentioned above.

Degradation of Galα(1-3)Gal. A stock solution of 16 mM digalactoside in 0.1 M sodium citrate buffer pH 5.5 was prepared, 20 µl of this solution were mixed with 100 µl of the enzyme solution and 300 µl of sodium citrate buffer. The reaction was carried out at 34°C and released free galactose was detected by liquid chromatography.

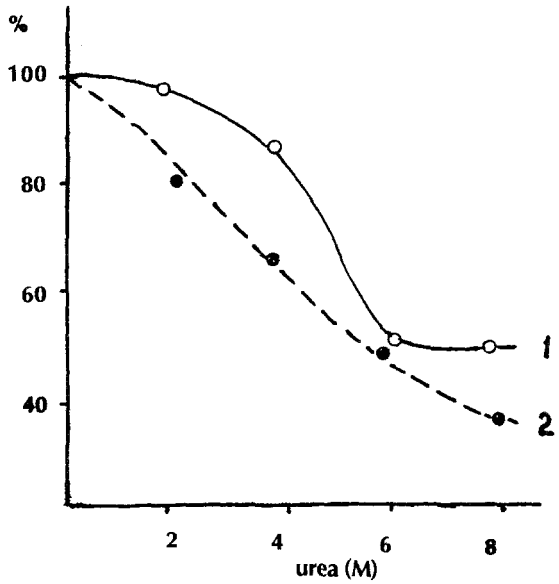


Figure 1. Effect of urea on α-galactosidase activity. Enzyme samples were incubated in the presence of urea in 0.1 M sodium citrate buffer pH 5.5 at 18°C. After 1 h (1) and 3 h (2), samples were removed and assayed for enzyme activity.

Degradation of a Galactomannan. The enzyme solution was incubated at a room temperature for 96 h with a galactomannan solution (2 mg/ml). The reaction was stopped by the addition of three volumes of ethanol; after centrifugation the supernatant was evaporated and free galactose was detected using liquid chromatography.

Protein Determination

Protein was determined by the methods of Lowry *et al.* [12] and Wolf [13].

Amino Acid Analysis

Samples of the purified enzyme were hydrolyzed for 24 h at 105°C in 6 N HCl by the method of Moor and Stein [14]. The total amino acid content of the α-galactosidase was determined with Liquimat III amino acid analyser (Labatron, W. Germany) as suggested in the manufacturer's manual. Tryptophan was not determined.

Sugar Analysis

The sugar composition of the purified enzyme was determined using gas-liquid chromatography. The molar ratios of neutral monosaccharides, hexosamines and sialic acid were determined after methanolysis with 1 N HCl in anhydrous methanol at 100°C during 24 h followed by N-acetylation. After neutralizing by drying in rotary evaporator, samples were treated with Sylon, dried in the atmosphere of N_2, dissolved in hexane and sugars were tested as trimethylsilyl derivatives [15]. Mannitol was used as internal standard. Chromatography was carried out using Hewlett-Packard 5840-A and 5710-A high performance chromatograph with FID detector. A capillary column (35 x 0.2 mm) was packed with 3% SE-30. The

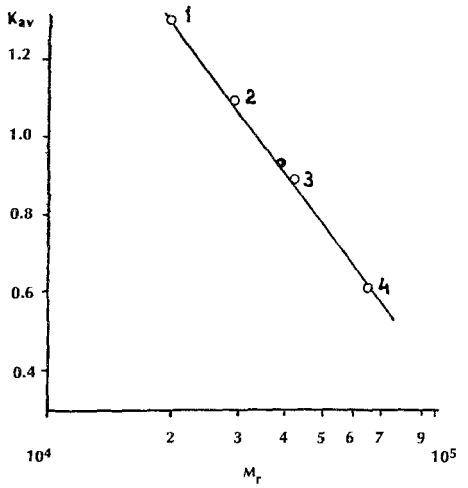


Figure 2. Estimation of molecular weight by Sephadex G-100 gel filtration after SDS-treatment. Standard proteins: 1, soybean trypsin inhibitor (20,100); 2, carbonic anhydrase (30,000); 3, ovalbumin (43,000); 4, bovine serum albumin (67,000). Details of the procedure are given in the Materials and Methods section.

He_2 flow rate was 40 ml/min and column temperature increased from 120° to 220°C with a temperature gradient of 4°C/min after the first 2 min at a constant temperature of 120°C). The standard equimolar mixture consisted of L-fucose, D-mannose, D-glucose, mannitol, D-ribose, D-xylose, L-arabinose, L-rhamnose, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine and *N*-acetylneuramic acid. The total amount of neutral sugars were also determined by the method of Dubois *et al.* [16].

Sodium Dodecylsulfate Gel Filtration

The molecular weight of α-galactosidase subunits was determined by analytical gel filtration following the method described by Osterman [17]. In this case a Sephadex G-100 column (2.0 x 55 cm) equilibrated with 0.02 M sodium phosphate buffer pH 6.0 was used after it had been calibrated with the Calibration Kit (Pharmacia Fine Chemicals, Uppsala, Sweden). Both α-galactosidase and standard proteins were denatured by incubation for 10 min at 100°C in 100 µl of the same buffer, containing 2.5% SDS (Sigma) and 5% β-mercaptoethanol (Loba-Chemie, Austranal Präparate, Austria). Standard proteins (soybean trypsin inhibitor, carbonic anhydrase, ovalbumin and bovine serum albumin) and α-galactosidase were monitored by their absorption at 280 nm with an LKB Monitor.

Hemagglutination Assay

Freshly drawn blood (human or rabbit) was obtained from healthy donors. Erythrocytes were prepared by removing the plasma and buffy coat and by washing four times with PBS pH 7.3. Rabbit erythrocytes were preliminarily treated according to the method of Mehta [18]

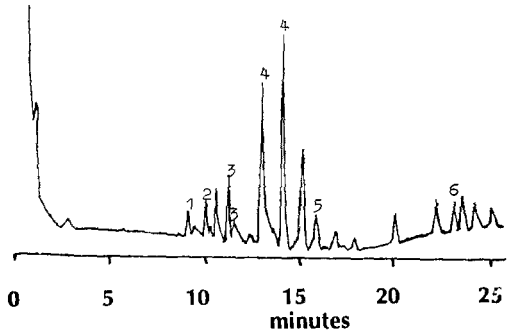


Figure 3. Gas-liquid chromatographic analysis of trimethylsilyl glycosides.
1, D-mannose; 2, D-galactose; 3, D-glucose; 4, mannitol; 5, *N*-acetyl-D-glucosamine; 6, *N*-acetylneuraminic acid. Further details are given in the Materials and Methods section.

with trypsin, chymotrypsin (both crystalline from Serva, W. Germany), Pronase, ficin (both from Calbiochem, San Diego, CA, USA), papain (Loba Chemie) or sialidase (Serva). The erythrocyte cells were fixed by glutaraldehyde (Reanal, Hungary) at its final concentration of 0.02% in PBS at 4°C. A 2% (by vol) suspension of erythrocytes or suspension with A_{620}=1.5 was used.

The reaction was performed at 4°C, erythrocytes were added to the enzyme samples with or without sugar that was preliminarily incubated in polystyrene plates (Linbro, USA) for 2 h. The "clot-dissolving" activity [19, 20] was tested after incubation of the enzyme samples with human B red cells for 40 min at room temperature. The titer of hemagglutinin activity was recorded as a minimal α-galactosidase concentration (μg of protein per ml) that caused agglutination [6]. One hemagglutinin unit was the amount of protein in the serial dilution of enzyme which corresponded to the titer.

Results and Discussion

Oligomeric Nature of α-Galactosidase

The loss of enzyme activity caused by urea is shown in Fig.1. After 3 h incubation at a urea concentration of 6 M it retains only 50% of the initial activity. It has been possible to establish that although the α-galactosidase inactivation is proportional to the concentration of urea, the exposure time was not critical. When the molecular weight of SDS-treated α-galactosidase was compared to those of proteins of known molecular weight (Fig. 2) it appeared to have a molecular weight of 40,000. However, it must be pointed out that due to the glycoprotein origin of α-galactosidase (see below) it is possible that this value does not

Table 1. Amino acid composition of α-galactosidase.

Amino acid	μmol amino acid /100 μg protein	Molar %
Asp	0.224	12.87
Thr	0.213	12.24
Ser	0.161	9.25
Glu	0.206	11.87
Pro	0.094	5.43
Gly	0.099	5.69
Ala	0.09	5.2
Cys	0.008	0.5
Val	0.067	3.82
Met	0.015	0.86
Ile	0.047	2.72
Leu	0.113	6.49
Tyr	0.07	3.74
Phe	0.063	3.62
Lys	0.17	9.74
His	0.058	3.3
Arg	0.062	3.59

reflect the true molecular weight of a subunit. The same conclusions is true for other α-galactosidases of microbial origin such as *Aspergillus tamarii* with subunits of M_r 88,000 and 77,000 [21], *Saccharomyces carlsbergensis*; 100,000 and 90,000 [22] and *Pycnoporus cinnabarinus*; 52,000 [23].

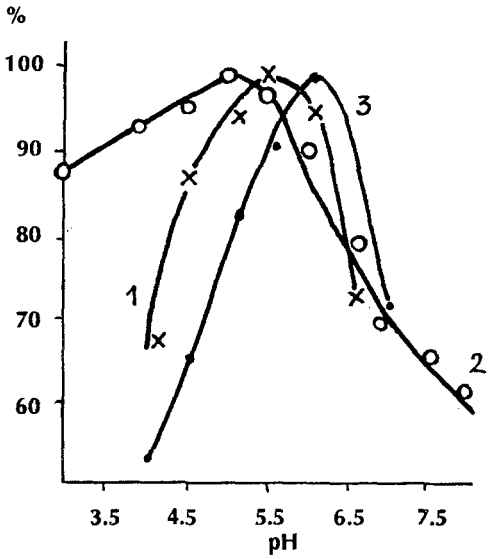
From these experiments we conclude that although the *C. acremonium* α-galactosidase is oligomeric, the determination of the complete quaternary structure after removal of the carbohydrate requires further investigation.

Glycoprotein Nature of α-Galactosidase

Amino Acid Composition. The amino acid composition of purified α-galactosidase is given in Table 1. About 33% of the residues can be accounted for by hydrophobic amino acids and about 21% by oxyamino acids. A quarter of the residues were represented by aspartic and glutamic acids. Compared with intracellular α-galactosidase of *Escherichia coli* [24] the enzyme from *C. acremonium* contains more threonine (about 12%) residues but only 5.2% of alanine and 6.49% of leucine residues. No other information concerning the amino acid composition of mold α-galactosidase was found. For the yeast *Sacch. carlsbergensis* [22] it was found that like the *C. acremonium* enzyme, the content of aspartic acid, glutamic acid and threonine was high (about 29%) in the α-galactosidase molecule.

Sugar Composition. As was described previously [5], during purification of α-galactosidase, evidence was accumulated for the glycoprotein nature of the enzyme. The total amount of neutral sugars determined by the method of Dubois was about 27% (By weight). The results obtained by gas-liquid chromatography (Fig. 3) gave *N*-acetylglucosamine, mannose, galactose and sialic acid in the molar proportions 2:7:3:11. Purified mycelial α-

(a)



(b)

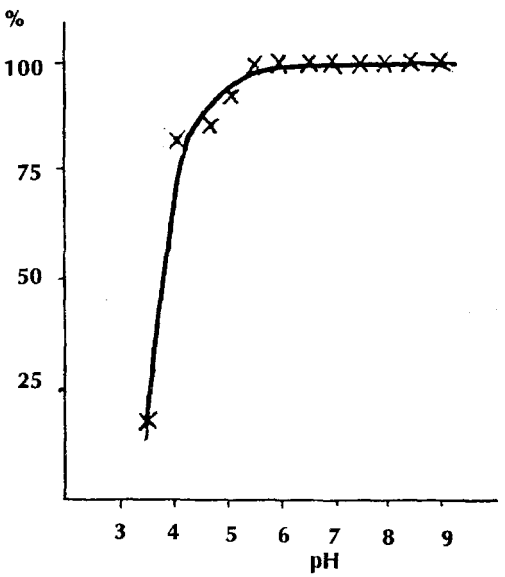


Figure 4. Effect of pH on α-galactosidase activity (a) and on the stability (b) of the enzyme. The assays were carried out with 0.1 M sodium citrate buffer from pH 3.0 to 6.5 and 0.1 M sodium borate buffer from pH 7.0 to 9.0. For other assay conditions see the text.

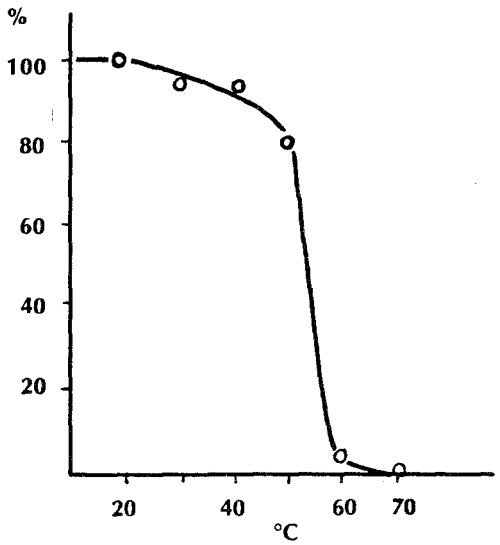


Figure 5. Thermal inactivation of α-galactosidase. Enzyme samples in 0.1 M sodium citrate buffer pH 5.5 were maintained for 20 min at the given temperature and suitable samples were withdrawn and assayed as usual with p-NPG as substrate.

galactosidase from *Asp. tamarii* examined by gas-liquid chromatography gave an *N*-acetylglucosamine, mannose, glucose and galactose molar proportion of 1:9:5:8 [21]. The secreted enzyme from the same culture contained *N*-acetylglucosamine, mannose and galactose in the molar proportion 1 : 6 : 1.5 [25]. The enzyme from *Asp. niger* contained mannose and glucosamine in the molar proportion 3:1 [26]. The carbohydrate portion of the extracellular yeast α-galactosidase from *Sacch. carlsbergensis* consisted of about 93% mannose, 7% glucose and 1% glucosamine [27]. The glycoprotein origin of the enzyme was also demonstrated for *Sacch. cerevisiae* var. *oleaceus* α-galactosidase which was found inside the plasma cell membrane [28].

Thus mannose and glucosamine are sugars present in all microbial α-galactosidases whose sugar composition was studied. These results obtained for *C. acremonium* α-galactosidase are the first data concerning *N*-acetylneuramic acid in the carbohydrate portion of this enzyme. However, according to Dean and Sweeley, sialic acid residues appear to be present in α-galactosidase from human liver, since changes in the pI, electrophoretic mobility and chromatographic properties of the enzyme have been observed following treatment with *Clostridium perfringens* neuraminidase [29]. Although we were not able to obtain a sufficient amount of the enzyme for detailed identification of the carbohydrate structure, a certain type of structure can be proposed based on the lectin chromatography data [5] and sugar analysis of *C. acremonium* α-galactosidase. It seems likely that the following Asn-linked structures are presented in the enzyme molecule: a) bi-antennary oligosaccharide of a complex type (that follows from the binding of the enzyme with LCA and the absence of such interaction with PHA) and b) an oligomannoside type structure (that follows from the

Table 2. Substrate specificity of α-galactosidase.

Substrate	V_{max} [a]	K_M [b]
p-nitrophenyl-α-D-galactopyranoside	4.5	0.7
melibiose	0.9	7.81
raffinose	25.5	100
methyl α-D-galactopyranoside[c]	hydrolyzed	
p-nitrophenyl β-D-galactopyranoside	not hydrolyzed	
p-nitrophenyl α-D-glucopyranoside	not hydrolyzed	
p-nitrophenyl β-D-glucopyranoside	not hydrolyzed	
p-nitrophenyl α-D-mannopyranoside	not hydrolyzed	
p-nitrophenyl β-D-mannopyranoside	not hydrolyzed	
p-nitrophenyl α-L-fucopyranoside	not hydrolyzed	
p-nitrophenyl β-D-xylopyranoside	not hydrolyzed	
p-nitrophenyl *N*-acetyl-α-D-galactopyranoside	not hydrolyzed	
p-nitrophenyl *N*-acetyl-β-D-glucopyranoside	not hydrolyzed	

[a] V_{max} is expressed as mM of substrate hydrolyzed per min per mg of protein.
[b] K_M is expressed as mM.
[c] Assays were by the reducing sugar method.

strong interaction with ConA) with regards to sugar to protein ratio α-galactosidase of *C. acremonium* may also contain more than one lactosamine-type and one oligomannoside-type chain. Moreover a slight interaction of α-galactosidase with WGA could be explained by the cluster situated sialic acid units.

Kinetic Properties of the Purified Enzyme

Optimal pH and Stability. The effect of pH on the α-galactosidase activity was studied at 37°C with p-NPG, melibiose and raffinose as substrates. Fig. 4a shows the enzyme activity measured as a function of pH obtained for these substrates. With p-NPG the maximal activity was found at pH 5.5 when melibiose and raffinose were used the maximal enzyme activity were obtained at pH 5.0 and 6.0, respectively.

The stability of α-galactosidase at various pH values was investigated by incubating the enzyme for 72 h at pH values from 3.0 to 9.0 at room temperature. As shown in Fig. 4b, the enzyme was stable at neutral and alkaline pH, however it became unstable below pH 5.0-5.5.

The enzyme was incubated at different temperatures from 20°C to 70°C in 0.1 M sodium citrate buffer pH 5.5 and the percentage of enzyme activity remaining was calculated. Fig. 5 shows that the enzyme was unstable and the activity was almost lost at 60°C. The purified α-galactosidase was unstable upon freezing and thawing and also on freeze-drying. When a concentrated preparation in 0.02 M sodium phosphate buffer pH 6.0 (about 5 mg per ml) was stored at 4°C no appreciable decrease in enzyme activity was found after 10 months. Being a glycoprotein, as was shown above, α-galactosidase from *C. acremonium* binding to ConA formed an active complex that is much more heat stable than the enzyme alone (Fig. 6). In the presence of methyl-α-D-mannoside the stabilizing effect of the lectin decreased

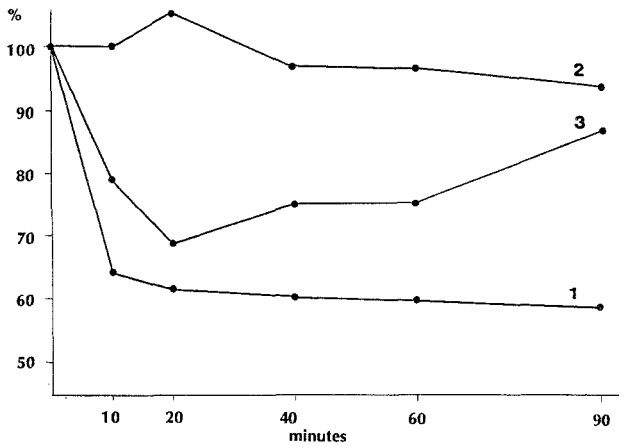


Figure 6. The effect of ConA on the thermal inactivation of α-galactosidase.
1, α-galactosidase; 2, α-galactosidase in the presence of ConA (100 μg/ml); 3, α-galactosidase in the presence of ConA and 0.2 M methyl-α-D-mannoside.

during the first 20 min of incubation, but then a continual increase of residual enzyme activity was observed (up to 86% for a period of 1.5 h). The activation energy determined from an Arrhenius plot was calculated to be 1701 cal per mol. This value is comparable to the 5400 and 5200 cal per mol reported for *Sacch. cerevisiae* var. *oleaceus* α-galactosidase [28].

Substrate Specificity and Enzyme Kinetics. The various kinds of synthetic glycosides listed in Table 2 were examined with a purified enzyme solution and the highly purified α-galactosidase from *C. acremonium* was demonstrated to have no enzymic activity on these substrates except p-NPG. The mold α-galactosidase cleaved the terminal α-galactosyl residues from methyl-α-D-galactoside, 4-MUG, galactosyl-α-(1-3)-galactose, melibiose and raffinose. The effect of substrate concentration on activity was examined by using both p-NPG, melibiose and raffinose. Lineweaver-Burk plots of the data are given in Table 2. For application of the enzyme to structural studies of glycoconjugates, hydrolysis of ceramide trihexoside, blood group B active substance and galactomannan from *Lagonichium farctum* by the enzyme was studied.

From the data obtained it could be concluded that the α-galactosidase from *C. acremonium* liberates the terminal galactose units not from only artificial substrates but also from oligosaccharides, glycoproteins and glycolipids (Table 3). This suggests a broad aglycone specificity of the enzyme, the hydrolysis of Galα(1-3)-, Galα(1-4)- and Galα(1-6)-linkages.

Clostridium sporogenes, Shigella flexneri, Streptomyces sp. and *Candida javanica* α-galactosidases have been reported to convert human type B erythrocytes to type O by cleaving the α(1-3) non-reducing terminal α-galactosyl units [30-33]. Treatment by α-galactosidase from *Asp. niger* (Sigma, USA) lead to a significant reduction of agglutinability of rabbit erythrocytes by human B antisera, as was shown by Pfannschmidt and Schauer [34]. However, the *Mort. vinaceae* α-galactosidase does not liberate D-galactose from group

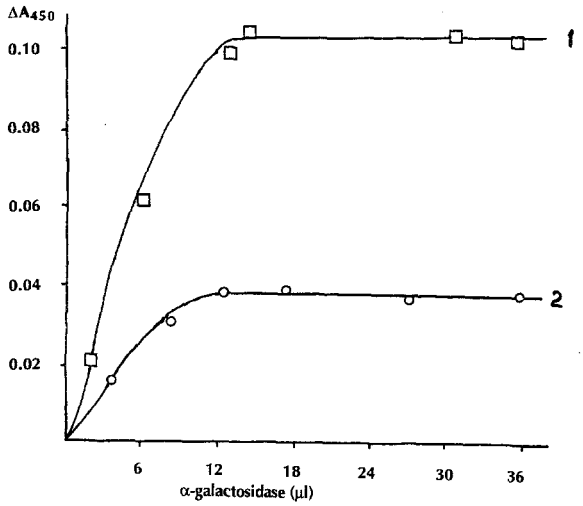


Figure 7. Agglutination of starch granule by α-galactosidase at 405 nm (Spectrophotometer Beckman 35, USA). 1, agglutination of starch suspension (A_o -1.7) in PBS pH 7.4 by different amounts of α-galactosidase (14 mg/ml); 2, agglutination of the same starch suspension by α-galactosidase in the presence of 0.25 M methyl-α-D-glucoside. $A_\Delta = A_o - A_i$, where A_i is the optical density of the starch suspension after the addition of α-galactosidase.

B substance [27]. Compared with the α-galactosidase of higher plants the substrate specificity of the microbial enzyme has almost been neglected. Any other data concerning the action of α-galactosidase of microbial origin on α(1-4) galactose units of ceramide trihexoside have not been found. Thus, the *C. acremonium* enzyme may be valuable for structural and functional studies of galactose-containing glycoconjugates.

It is a well known fact that a number of plant α-galactosidases possess also lectin (hemagglutinin) activity. Lectin activity of the *C. acremonium* enzyme was detected in various erythrocyte systems with up to 1 μg of the enzyme during 2 h incubation at 4°C. A number of carbohydrates (D-mannose, D-glucose, L-fucose, D-fucose, L-arabinose, inositol, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, methyl-α-D-glucopyranoside, methyl-α-D-mannopyranoside, maltose and melibiose) were examined for their ability to inhibit the agglutination reaction. The inhibition studies were carried in PBS pH 7.4 (with α-galactosidase of 4 HU). The only compounds that showed significant inhibitory effect were D-glucose, methyl-α-D-glucopyranoside and methyl-α-D-mannopyranoside. It is of interest that the lectin activity is not inhibited by D-galactose, or melibiose, p-NPG and methyl-α-D-galactoside.

The agglutination of a starch granule suspension by *C. acremonium* α-galactosidase measured by the turbidimetric technique was also studied. In Fig. 7 a bell-shaped curve, typical of a lectin-polysaccharide interaction, of the protein concentration dependence is shown. In the presence of 0.25 M methyl-α-D-glucopyranoside (Fig. 6, curve 2), which was

Table 3. Action of α-galactosidase on various galactosides.

Substrate	Units of enzyme	Incubation time (h)	Galactose released (nmol)
Galα(1-3)Gal	20	0.25	7.2
Blood group B substance	61.4	3	27.5
Ceramide trihexoside	47.5	24	0.9
Galactomannan	5	96	1.7

The details are given in the Materials and Methods section.

found to be the inhibitor of the hemagglutinin reaction, the maximal measured reduction of the α-galactosidase-starch interaction was 64%. Under the same conditions the α-galactosidase precipitated also α-glucans of the dextran type (T-20, T-40, T-500; unpublished data).

Thus the lectin activity of *C. acremonium* α-galactosidase is specific for glucose (mannose), whereas the catalytic activity is specific for galactose. When tested on blood group B erythrocytes, the *C. acremonium* α-galactosidase-specific hemagglutinin activity also displays "clot-dissolving" activity, a phenomenon which has been described in detail elsewhere [1, 19]. The carbohydrates mentioned above differed also by their ability to inhibit the "clot-dissolving" activity of the enzyme studied [6].

The evidence for the existence of separate catalytic and lectin sites on the enzyme molecule came from investigations on the effect of pH on the three activities, whereas the optimum pH for catalytic activity was found to be at 5.5 (for p-NPG), the pH optimum for the "clot-dissolving" activity was at 7.1 and the titer of hemagglutination activity of α-galactosidase increased with increasing pH value. At pH 8.3 α-galactosidase was able to agglutinate human B erythrocytes at a concentration of 0.1 μg/ml. Our data indicate that *C. acremonium*, a representative of phytopathogens, secreted an α-galactosidase which has much in common with the enzyme from legume species [1, 2]. The lectin activity of other microbial α-galactosidases has not yet been studied. α-Galactosidase is shown to be a glycoprotein-lectin and therefore represents an interesting system of a considerable potential physiological importance. In *C. acremonium* the enzyme is located in the periplasma and is believed to be closely associated with the cell wall [35]. The cell wall contains enzymes which are primarily hydrolases whose function is to hydrolase substrates which would otherwise not cross the cell membrane. Both the lectin sites and carbohydrate chains of α-galactosidase may be important for binding of the enzyme to plant cell components *via* lectin interactions. Such bonds could conceivably regulate the activity of the catalytic site of α-galactosidase.

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