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Glucoamylase from the Predacious Fungus *Arthrobotrys conoides*: a Cationic Enzyme with High Debranching Activity and Raw Starch Digestibility[1]

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Abstract—The extracellular amylolytic activity elaborated by the nematophagous fungus *Arthrobotrys conoides* was found to resolve into 2 amylolytic peaks when fractionated on Sephadex G-100 column. Around 80% of the eluted glucoamylase activity was attributed to peak I (GA A). GA A being cationic in nature was purified about 70-fold with 57% yield by negative chromatography on DEAE Sephadex at pH 7.0. The enzyme was stable over a broad pH range of 4.8–9.0. K_M for the linear polysaccharide amylose was 0.34 mg/mL. Enzyme showed high affinity for the branched polysaccharides as the K_M values for amylopectin, glycogen and starch were 0.056, 0.062 and 0.065 mg/mL, respectively. The enzyme clearly demonstrated raw starch digestibility. Probable involvement of Trp and His residues in enzyme catalysis was elucidated using group-specific reagents.

Keywords: glucoamylase, *Arthrobotrys conoides*, purification, debranching activity, raw starch digestion

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The genus *Arthrobotrys* is a group of predacious fungi, which kill and consume microscopic animals. In the environment, they are predominantly found in leaf compost, decayed wood or plant roots. In presence of organic nutrients, these organisms thrive as successful saprophytes. At a later stage, when substrate level depletes, the organisms turn predacious [1]. These fungi are known to produce collagenases, pectinases, amylases, cellulases and chitinolytic enzymes. One of the interesting fields in research is to study the potential of these carnivorous fungi for use as biocontrol agents against pathogenic nematodes [2–5]. Not much information is available on the enzymes elaborated by *Arthrobotrys* species. In our previous study, we reported production of extracellular glucoamylase (GA) from *Arthrobotrys conoides*. Glucoamylases (EC 3.2.1.3.) are exo-1,4-α-glucan glucohydrolases which act on glucan polysaccharides yielding glucose as the major product. GAs are extensively employed in production of glucose syrup, high fructose corn syrup and alcohol. Some GAs are also known to hydrolyse α-1,6 and α-1,3 linkages albeit at a much slower rate. Raw starch digestibility and high debranching activity adds to the desirability of amylolytic enzymes in industrial applications. Identification of GA- producing organisms, the production, purification and kinetics of GAs have gained interest as the ability of amylases to hydrolyse starch and maltodextrins finds applications both in natural and industrial amylolytic processes [6–9].

The aim of the present investigation was to purify and characterize the extracellular amylolytic enzyme produced by the nematophagous fungus *A. conoides*. The purified enzyme was treated with periodate and the modified enzyme was also characterized to study the significance of glycosyl residues in enzyme action.

MATERIALS AND METHODS

Microorganism. The organism *A. conoides* was procured from American Type Culture Collection, Maryland, USA. The culture was maintained on corn meal agar slants as recommended by ATCC catalogue, 1983.

Enzyme production. Enzyme was produced in a medium containing (g/L): corn starch—10.0, peptone—2.0, $NaNO_3$—3.7, KH_2PO_4—3.4, KCl—0.5 and $MgSO_4 \cdot 7H_2O$—0.5; (mg/L): $FeSO_4 \cdot 7H_2O$—10.0, $ZnSO_4 \cdot 7H_2O$—0.5, thiamine HCl—0.1 and (μg/L): biotin—5.0 (pH 7.2). The medium (25 mL) was dispensed into 250 mL Erlenmeyer flasks and inoculated with around 10^6 fungal spores. Incubation was carried out at 25 ± 1°C for 12 days under stationary conditions. After filtration through Whatman No. 1 filter

[1] The article is published in the original.

paper (Fischer Scientific, UK), the culture filtrate was used for further studies.

Enzyme purification. The culture filtrate was dispensed into diaylsis bags and concentrated against PEG 20000. Further concentration was carried out by 80% ammonium sulfate saturation at 4°C. Concentrated enzyme (≈50 units) was loaded on 1.6 × 65 cm Sephadex G-100 column pre-equilibrated with 50 mM acetate buffer (pH 5.6). Fractions of 2 mL were collected at a flow rate of 3 mL/h. The active GA fractions obtained from peak I were pooled, concentrated and then loaded on a DEAE Sephadex A-50 column (1.6 × 20 cm) and run was carried out in 50 mM phosphate buffer (pH 7.0). Elution of the adsorbed enzyme was performed by applying a linear gradient of 0–1.5 M NaCl in the same buffer.

Analytical methods. Amylolytic activity was measured in 2 mL of the reaction mixture containing appropriately diluted enzyme and 0.25% soluble starch in 25 mM acetate buffer (pH 5.6). After incubation at 50°C for 20 min, the resultant reducing sugars were measured by dinitrosalicylic acid (DNSA) method [10].

Glucose produced was estimated by using glucose oxidase-peroxidase chromogen reagent (GOP method) [11]. Unit of GA activity was defined as the amount of enzyme which hydrolyzes soluble starch to release 1 μmole of glucose per min under the assay conditions.

Protein was estimated using Folin's phenol reagent.

Determination of carbohydrate content: to 0.5 mL of 5% (wt/vol) phenol solution 0.5 mL of appropriately diluted sample was added followed by gentle addition of 2.5 mL of concentrated sulfuric acid. The tubes were left undisturbed for 10 min at 30 ± 1°C. Then the contents were mixed and incubated for 15–20 min at the same temperature and absorbance was read at 490 nm.

Electrophoresis. Native PAGE was carried out with 7.5% separating gel and 3.1% stacking gel. To stain protein, the gel was immersed in 12% trichloroacetic acid for 30 min followed by staining with 0.125% Coomassie Brilliant Blue R-250 at 30 ± 1°C for 45 min. Enzyme activity was localized on the gel by immersing in 1% soluble starch solution (in 0.1 M acetate buffer, pH 5.6) at 50°C for 30 min. Gels were then stained with iodine solution (0.127% I_2 and 0.254% KI) at 30 ± 1°C for 5 min. Enzyme activity was localized as zone of clearance in the dark (bluish-black) gels.

Molecular weight determination. Molecular mass of GA from *A. conoides* was determined by gel filtration in Sephadex G-100 column using molecular weight markers (MF-GF-70, Sigma) and by SDS-PAGE (12% T, 5% C) according to Laemmli's method.

Characterization of the GA from *A. conoides*. *Effect of temperature and pH.* Effect of pH and temperature on the activity of the GA from *A. conoides* was studied. Enzyme was incubated at 30°C for 1 h in various buffers (pH 4 to 12.5; 50 mM acetate, phosphate or NaOH-glycine) for pH stability studies. Residual activity was then assayed. Thermal stability was tested by incubating the enzyme at temperatures ranging from 40–55°C for different time periods up to 60 min in 25 mM acetate buffer (pH 5.6).

Effect of metal ions and acarbose. Effect of acarbose, $HgCl_2$ (0–0.25 mM), EDTA, $CaCl_2$, $CuCl_2$, lead acetate, $ZnCl_2$, $MnCl_2$ and $MgCl_2$ (0–5 mM) was studied. GA A was incubated with the reagents at 25°C for 30 min and then assayed.

Effect of group-specific reagents. N-ethyl maleimide (NEM) and iodoacetate were used at a range of 0–3 mM as Cys-specific reagents. Glyoxal (0–3 mM), pyridoxal phosphate (PALP; 0–3 mM), diethyl pyrocarbonate (DEP; 0–15 mM) and N-bromosuccinimide (NBS; 0–10 μM) were used as group-specific agents for Arg, Lys, His, Trp, respectively. DEP stock (0.3 M) was prepared in ethanol. Effect of NEM, DEP and the other reagents was investigated at pH of 6.0, 7.0 and 8.0, respectively. Respective amino acids were used as quenching agents to neutralize unreacted reagent. One mL of reaction mixture containing GA A, and the amino acid group-specific agent in 0.075 M phosphate buffer at the reagent-specific pH was incubated for 30 min at 10°C at 100 rpm, followed by the addition of 0.1 mL of 50 mM quenching agent (1 mM in case of Trp). In all the tubes the group-specific reagent was brought to the highest concentration used, and assayed for amylolytic activity by DNSA method.

Effect of Rose Bengal (His-specific reagent) was studied by immersing a vial containing the enzyme and Rose Bengal (0.25 mg/mL) in a chilled water bath maintained at 5–10°C. DTT was added to a final concentration of 1 mM. The reaction mixture was irradiated using a 200 W lamp placed at a distance of 6 cm. Aliquots were taken at various time intervals ranging from 0–45 min, and the amylolytic activity was measured by DNSA method.

Substrate protection against NBS and DEP. In substrate-protected samples, the enzyme was pre-incubated with starch (at pH 7.0 for DEP and pH 8.0 for NBS). The mixture of 10 μM NBS and 15 mM DEP was added and incubated at 15°C for 10 min. Respective quenching agent was added, followed by further addition of substrate starch and amylolytic assay was carried out. Appropriate controls were maintained for both protected and unprotected GA A.

Periodate treatment. GA A preparation in 50 mM acetate buffer (pH 5.6) containing 42 U/mL was treated with sodium metaperiodate of various concentrations (0–12 mM) for 15 min. The reaction mixtures were then dialyzed overnight against 5 mM acetate buffer (pH 5.6).

Raw starch digestion. Suspension of 0.5% agar in 25 mM acetate buffer (pH 5.6) was solubilized in boiling water bath. After cooling to 50°C, 0.5% cornstarch was added and dispensed (17 mL) into Petri plates

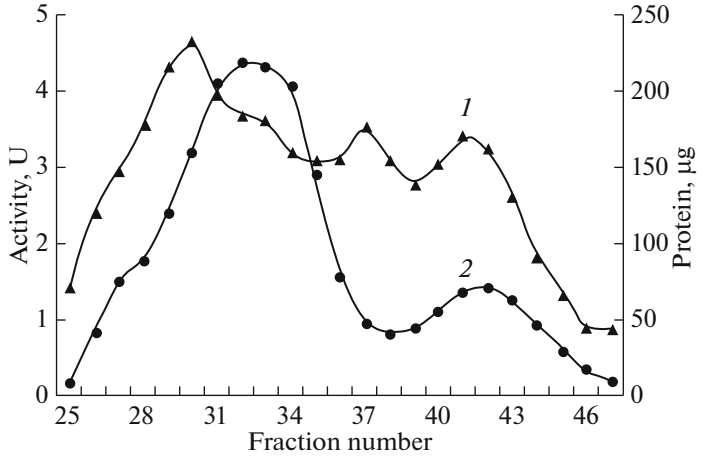

Fig. 1. Gel filtration profile of the crude GA from *A. conoides* on Sephadex G 100. *1*—Protein; *2*—GA activity.

(diameter of 9 cm) for preparation of raw starch agar plates. Petri plates containing 0.5% gelatinized cornstarch agar were also prepared. Wells (diameter of 0.6 cm) were bored in each plate. The GA fractions from *A. conoides* were loaded into the wells. After 5 h of incubation at 37°C, the plates were flooded with 0.125% I_2 solution. Amylolytic activity was observed as a zone of clearance around the wells.

Raw starch adsorption and digestibility studies. Corn starch (0.2 g) was washed twice with 0.1 M acetate buffer (pH 5.6). To the starch pellet, 3 mL of enzyme solution containing from 0.5 to 2 U was added. The suspension was incubated at 10°C for 30 min at 100 rpm and then centrifuged at 2600 g at 4°C for 10 min. Supernatant was collected. The pellet was washed twice with 50 mM acetate buffer (pH 5.6) and the washings were pooled with the respective supernatant. Supernatant was assayed to determine the unadsorbed activity. To the washed pellet, 3 mL of the same buffer was added, mixed well and incubated at 37°C in a shaker water bath for 6 h and centrifuged under the same conditions. The glucose released was estimated by GOP method.

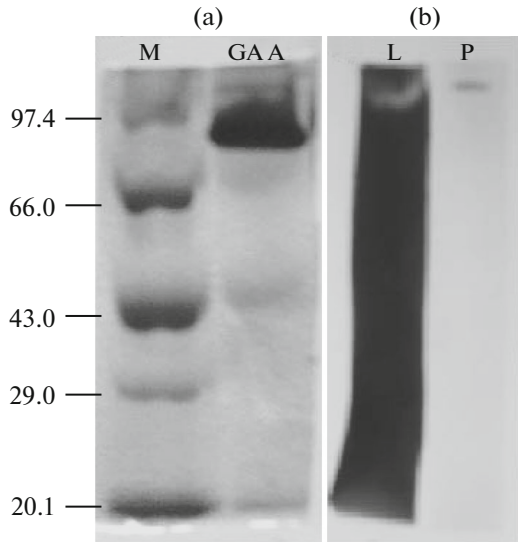

Fig. 2. SDS-PAGE (a) and native PAGE (b) of GA from *A. conoides*. (a) Lane M—molecular weight markers; (b) lane L—localization of the enzyme activity; P—protein staining.

RESULTS AND DISCUSSION

A. conoides was found to produce 2.4 U/mL of amylolytic activity. Nature of the amylolytic activity of the fungus was studied by estimating the starch hydrolysis products by both DNSA and GOP methods. Ninety five percent of the reducing sugars were found to be glucose. The result is in accordance with the report of Jaffer et al. [12] in which the descending paper chromatography of the reaction products of amylolytic activity produced by various *Arthrobotrys* species showed that major product of hydrolysis was glucose.

The culture filtrate was concentrated by dialyzing against PEG 20000 followed by salting out at 80% ammonium sulfate saturation. The concentrated enzyme resolved into 2 peaks on Sephadex G-100 column (Fig. 1). Fractions 26–36 of the major peak (peak I) and 39–46 of peak II were separately pooled. Peak I comprised around 81% of the total eluted GA activity. Peak I was further purified by chromatography on DEAE Sephadex A-50 at pH 7.0. It was found that 91.5% of the loaded activity did not bind to the anion exchanger and was hence, found in the flow-through. The fractions eluted by 0–1.5 M NaCl gradient showed negligible activity. The unbound enzyme purified by negative chromatography was designated as GA A. The result of purification is summarized in Table 1. GA A from *A. conoides* was purified 68-fold

with a specific activity of 166 U/mg of protein and an overall yield of about 57%.

The molecular mass of GA A as determined by gel filtration and SDS-PAGE was around 87.0 and 83.5 kDa, respectively (Fig. 2a). As the enzyme did not bind to DEAE Sephadex A-50 at pH 7.0, adsorption of GA A was studied at higher pH values. It was revealed that GA A did not bind to the matrix at pH 8.0 (binding was <12%); but at pH 9.0 and above, >82% of the activity was found to adsorb onto the matrix. The enzyme was subjected to non-denaturing PAGE at pH 8.3. Protein and the GA A activity bands could not be localized in the separating gel. Results of native PAGE performed at pH 8.8 are shown in Fig. 2b. The protein band and activity could be localized in the gel. However, it was found that the enzyme barely migrated into the separating gel, remaining almost at the top of the separating gel. Both the DEAE Sephadex A-50 binding experiments and native electrophoresis results proved that the GA A elaborated by the *A. conoides* was cationic in nature.

The pH optimum for the enzyme was found to be at pH 5.4–5.8 (Fig. 3). GA A exhibited an interesting and beneficial property of being stable in the alkaline region as well, exhibiting stability over a broad pH range of 4.8–9.0. The enzyme was found to be stable at 40°C for 30 min (results not shown). Residual activity after 1 h of incubation at 45°C was around 59%. However, the temperature optimum was found to be 50°C.

The K_M values of the GA from *A. conoides* for starch and the highly branched glucans are significantly lower than those reported for most other GAs [9, 13–18]. Figure 4 demonstrates high affinity of GA A for starch and other branched polysaccharides. The K_M value for amylose (linear polysaccharide) was 6 times higher than the K_M values for amylopectin and glycogen. The enzyme acted effectively on these branched polysaccharides. V_{max} values for starch, amylopectin and glycogen were 147, 137 and 125 U/mg of the protein respectively. Glycogen, the highly branched polymer, is present in appreciable amounts in nematodes as one of the important energy carbohydrate stores and comprises around 3–20% of dry weight [19, 20]. Ability of the enzyme to act on raw starch was demonstrated in agar plates containing the raw starch, wherein GA A showed clearance against

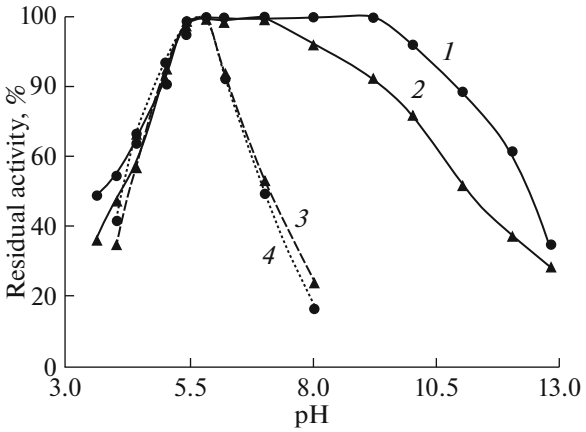

Fig. 3. Effect of pH on the activity and stabiity of the GA from *A. conoides*. *1*—GA A stability; *2*—GA AP stability; *3*—GA AP activity; *4*—GA A activity.

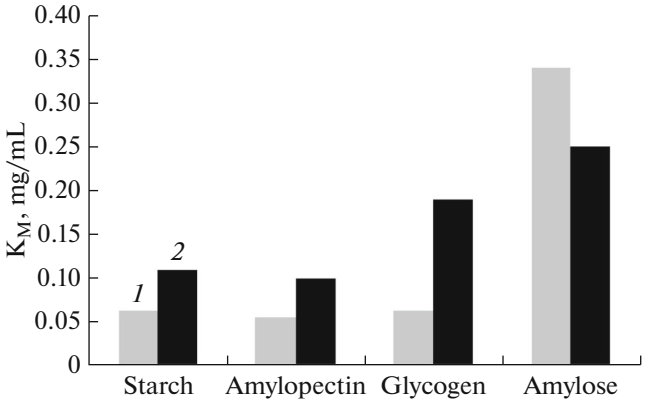

Fig. 4. Affinity of the GA from *A. conoides* for polysaccharide substrates. *1*—GA A; *2*—GA AP.

Table 1. Purification of GA from *A. conoides*

Step	Activity, U	Protein, mg	Specific activity, U/mg of protein	Purity, -fold	Yield, %
Cell free extract	148	61.0	2.43	1	100
Concentration					
PEG	136	31.2	4.35	1.79	91.89
80% ammonium sulfate	125	15.8	7.93	3.26	84.46
Sephadex G-100					
Peak I (GA A)	89.1	5.3	16.85	6.93	60.2
Peak II	20.9	2.7	7.80	3.21	13.12
DEAE Sephadex A-50					
GA A (flow through)	84.6	0.5	165.80	68.20	57.1

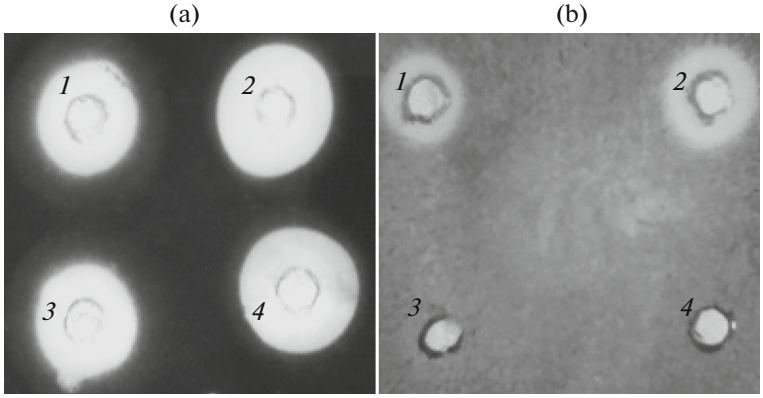

Fig. 5. Raw starch digestibility by the native and periodate-modified GA from *A. conoides.* (a) Effect on soluble starch; (b) effect on raw starch. *1*—0.15 U of GA A; *2*—0.30 U of GA A; *3*—0.15 U of GA AP; *4*—0.30 U of GA AP.

dark blue background when flooded with iodine (Fig. 5). The high affinity GA A from *A. conoides* for glycogen as well as high raw starch digestibility appears to be the characteristic of the fungus in its adaptation to saprophytic as well as nematophagous phases of life.

Influence of various group-specific reagents and metal ions on GA from *A. conoides* was investigated. The results of significance are summarized in Table 2. Acarbose (pseudotetrasaccharide) is a potent inhibitor of GAs. Involvement of 2 specific tryptophanyl residues in the reversible binding of acarbose to the GAs of fungal species has been suggested by Meagher et al. and Sauer et al. [21, 22]. GA A from *A. conoides* was also strongly inhibited by acarbose implicating the involvement of tryptophanyl residues in catalysis. The hypothesis was further supported when the enzyme was treated with NBS, a commonly used probe for Trp.

Table 2. Inhibition of GA A from *A. conoides* by various group-specific reagents and metal ions

Reagent/metal ion	Concentration	Activity retained, %
Acarbose	2 μg/mL	34.0 ± 2.0
	4 μg/mL	15.7 ± 1.0
DEP	4 mM	53.0 ± 2.4
	10 mM	24.0 ± 3.4
	15 mM	20.1 ± 1.6 *54.8 ± 2.4**
Rose Bengal	0.25 mg/mL, 20 min	51.8 ± 2.4
	0.25 mg/mL, 45 min	27.7 ± 2.4
NBS	0.002 mM	78.6 ± 2.1
	0.004 mM	51.2 ± 2.6
	0.010 mM	21.7 ± 2.2 *68.2 ± 1.6**
EDTA	2.0 mM	49.8 ± 2.1
Cu^{2+}	0.1 mM	57.0 ± 1.6
Pb^{2+}	2.0 mM	71.2 ± 1.7
Hg^{2+}	0.002 mM	53.9 ± 2.5
Zn^{2+}	2.0 mM	49.1 ± 1.4

* Activity retained in substrate-protected samples.

Glyoxal, iodoacetate/NEM and NBS are known to bind specifically/preferentially to amino acids Arg, Cys and Trp, respectively [23–27]. DEP and PALP are reactive towards His and Lys residues [28, 29]. Rose Bengal-sensitized photooxidation has also been employed to investigate the significance of His residues in enzyme action. [30]. NBS, DEP and Rose Bengal were found to inhibit GA activity from *A. conoides*. NBS oxidizes the indole group of Trp to an oxindole derivative and 10 μM NBS was able to cause 78% inhibition of enzyme activity. Substrate offered considerable protection to the enzyme against NBS action. In the presence of 0.6% starch, the inactivation of GA A by 10 μM NBS was around 32%. However, NBS is also known to react with amino acids such as Cys and Lys. As iodoacetate, NEM and PALP exhibited no effect, the involvement of these residues in the enzyme activity was ruled out. DTT and glyoxal showed negligible effect on GA activity from *A. conoides*. Disulfide linkages and Arg are hence not essential for the activity of this enzyme. Modification of His residues was studied using Rose Bengal, which inactivated GA A to an extent of 73% at a concentration of 0.25 mg/mL. Met, Cys and Trp are also known to react with this reagent, albeit to a lesser extent. DTT was added to photooxidised samples to reduce the oxidation products of Met and Cys. The possibility of inhibition being due to Met and Cys therefore did not exist. As already mentioned earlier, The Cys modifying agents, NEM and iodoacetate, also did not have any effect on enzyme activity. DEP was used for His modification to further confirm the results. It shows good specificity for His residues at pH near neutral value. DEP at a concentration of 30 mM inactivated GA A completely. At 15 mM, the enzyme retained

around 19% of activity as against 55% in presence of substrate. Trp and His, therefore, appear to play an important role in the catalytic action of the GA from *A. conoides*. Of the metal ions tested, divalent ions of Cu, Zn, Hg and Pb were found to inhibit the enzyme. Hg^{2+} was a potent inhibitor as it could inhibit the enzyme to an extent of 46% at 2 μM concentration. Calcium enhanced the activity by 23% at a concentration of 4 mM, while 2 mM EDTA inhibited 51% of the activity. Mg^{2+} and Mn^{2+} had negligible effect on enzyme activity.

GAs are reportedly glycoproteins in nature [31]. Carbohydrate content of GA from *A. conoides* as determined by phenol-sulfuric acid method was around 26.3%. Periodate is used for oxidative cleavage of the carbohydrate moieties in glycoproteins. The hydroxyl groups attached to the adjacent carbon atoms are selectively oxidized to aldehyde groups, facilitating fission of the intervening C-C bonds [32, 33]. Oxidation of GA A with increasing concentrations of periodate could be correlated to reduction in both activity and carbohydrate content of the enzyme (Fig. 6). Around 27% of the activity and 34% glycosyl content was lost at 2.1 mM of periodate. Glycosylation is known to confer stability to the proteins. Having established the effect of periodate on glycosyl content of the enzyme, the study proceeded to assess the significance of glycosyl residues in the functionality of GA A treated with 2 mM periodate (designated GA AP). The temperature activity/stability profile and pH optima of the enzyme activity remained almost unaltered. Optimum temperature was 50°C and both the native and GA AP forms retained 100% of activity at 35°C and lost 10% of activity at 40°C in 30 min. Both enzyme forms were optimally active at pH range of 5.4–5.8 (Fig. 3). The pH stability curves differed significantly from each other. Native form of the enzyme exhibited stability over a broad range of pH from 5.4 to 9.2 for 60 min. In contrast, GA AP retained its stability over a narrower range of pH from 5.4 to 7.0. Glycosylation appears to confer stability to the enzyme in alkaline conditions. As presented in Fig. 4, the affinity of the enzyme for different substrates was influenced significantly by the glycosylation status of the enzyme. Affinity for starch and the other two branched polysaccharides namely, amylopectin and glycogen,

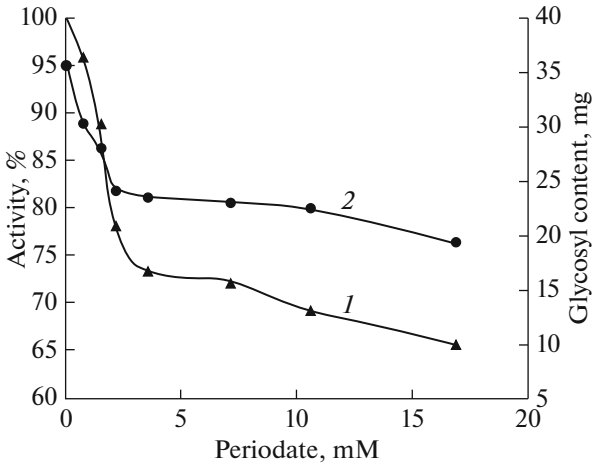

Fig. 6. Effect of periodate treatment on the activity and glycosyl content of GA from *A. conoides*. *1*—activity, %; *2*—glycosyl content/100 mg protein.

decreased significantly when treated with periodate. Interestingly, for amylose (linear polysaccharide) the reverse was true. K_M of GA AP for glycogen (highly branched substrate) increased almost 3 times, whereas for starch and amylopectin the increase was around 1.75-fold. It appears that glycosyl residues facilitate binding of the branched substrate to the enzyme.

Bisaria and Mishra have discussed the significance of glycosylation in cellulases [34]. It was suggested that additional hydroxyl groups at Ser and Thr residues due to glycosylation aid formation of hydrogen bonds with hydroxyl groups of cellulose molecule. Apparently, glycosylation plays an important role in binding of the enzyme to crystalline cellulose. Non-glycosylated forms of the enzyme retained activity with soluble substrate. GAs have functional similarities to cellulases in terms of cleavage of glycosidic linkages. The possibility of similarity to cellulases with respect to substrate binding mechanism may exist. The present investigation made an attempt to study the significance of glycosylation in raw starch digestion. GA AP was unable to act on raw starch (Fig. 6). The ability of the enzyme to bind to raw starch decreased significantly when treated with periodate (Table 3). More than 90% of native GA A could bind to raw starch and in case of

Table 3. Raw starch digestibility by GA A and GA AP from *A. conoides*

Units applied (A)	Unadsorbed activity, U (B)		Activity adsorbed, U (= A–B)		Glucose produced by bound enzyme, mg (total)	
	GA A	GA P	GA A	GA P	GA A	GA P
0.5	Neg*	0.44	0.50	0.06	9.30	0.94
1.0	0.07	0.91	0.93	0.09	15.30	1.64
1.5	0.13	1.34	1.37	0.16	21.86	2.47
2.0	0.20	1.64	1.80	0.36	26.78	3.14

* Negligible.

GA AP, approximately 90% of the activity remained unadsorbed. Consequently, the bound units of GA A produced 8–10-fold more glucose than bound GA AP. The experiment confirmed that glycosylation status of the enzyme has a critical impact on raw starch binding ability.

The dual nature of carnivorous fungi in its adaptation to both saprophytic as well as nematophagous phases of life has not received much attention from the scientific community. These fungi are reservoirs of hydrolytic enzymes. *A. conoides* produced a cationic GA which exhibited high debranching activity and raw starch digestibility. One of the negative aspects in GAs is its association with transglucosidase activity. Our enzyme was found to lack transglucosidase activity [12]. Thus, the enzyme exhibited interesting and promising properties.

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