

Full Length Research Paper

Characterization of a cellulase from the haemolymph of the giant African snail (*Archachatina marginata*)

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The giant African snails (*Archachatina marginata*) are herbivorous tropical gastropods. They are commonly located in areas littered with decaying vegetable matters which they feed on. When the haemolymph of this organism was passed through Sephadex G-200, the resin was digested. The ability of snails to digest vegetable matters and the digestion of Sephadex G-200 resin by the haemolymph led to the suspicion of the presence of cellulase in snail haemolymph. Cellulase from haemolymph of the giant African snail was purified to homogeneity by a combination of gel filtration on BioGel P-300 and ion-exchange chromatography on DEAE-Sephadex. The homogeneity of the pure enzyme was adjudged by polyacrylamide gel electrophoresis in the absence and presence of sodium dodecyl sulphate. The subunit molecular weight was $16,875 \pm 1,556$ daltons and the apparent molecular weight as determined by gel filtration was 52,000 daltons. Thus, the enzyme is at least a dimer. The enzyme had a specific activity of 1359.09 units/mg of protein. The Michaelis-Menten constant (K_m) for carboxymethyl (CM)-cellulose was 5.17 ± 0.74 mg/ml and the maximum velocity (V_{max}) was 1067 ± 195 units/ml. The enzyme did not degrade salicin, cellobiose and o-nitrophenyl- β -D-glucopyranoside. In addition, its activities towards filter paper and cotton wool were insignificant compared with its activity towards CM-Cellulose. Linamarin was found to inhibit the action of the enzyme to about 18%. The high specificity for CM-cellulose and the rapid decrease in the viscosity of CM-cellulose suggests that the enzyme is likely an endoglucanase.

Key words: Snail, cellulase, endoglucanase, haemolymph, gastropod.

INTRODUCTION

Cellulase is responsible for the hydrolysis of the β -1,4-glycosidic bonds in cellulose (a linear homo-polysaccharide composed of D-glucopyranose units), lichenin (a major polysaccharide component of Iceland moss, *Cetraria islandica*, composed of β -D-glucopyranose units linked in a linear manner by β -1,4- and β -1,3-glycosidic bonds) (Chandae et al., 1957; Peat et al., 1957; Pitiakoudis et al., 2003; Bala et al., 2008) and in cereal beta-D-glucans. Cellulase is an enzyme complex consisting of at least three components generally classified by their hydrolysing functions into the

following: endo- β -1,4-glucanase (EC 3.2.1.4) randomly hydrolysing the middle of cellulose chains, exo- β -1,4-cellobiohydrolase (EC 3.2.1.91) hydrolysing the non-reducing end of cellulose chains into a disaccharide, cellobiose units and β -glucosidase (EC 3.2.1.21) hydrolysing cellobiose or longer chains from non-reducing end to glucose units (Watanabe et al., 2001). The β -1, 4 linkages of cellulose are highly resistant to acid hydrolysis. It yields D-glucose with strong mineral acids and on partial hydrolysis yields cellobiose.

Cellulose is also not hydrolysed by glycosidases found in the digestive tracts of humans or other higher animals. Cellulase has been shown to exist not only in plants, mould, fungi, bacteria and protozoa, but also in herbivorous invertebrates such as arthropods, nematodes and mollusc (Suzuki et al., 1993). Snails and

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termites secrete an enzyme that hydrolyses cellulose, while ruminants are able to hydrolyse cellulose and utilise the glucose produced because the symbiotic bacteria in their rumen secrete cellulase(s) (Conn and Stumpf, 1976). Several different kinds of cellulase are known, which differ structurally and mechanistically. The applications of this enzyme in the industry are numerous. The enzyme is useful in the paper and agricultural industries. For examples, cellulase is used for commercial food processing in coffee, hydrolysis of cellulose during drying of beans, used in textile industry (making cotton cloths softer by limited hydrolysis (Woodward et al., 1994) and included in laundry detergents), pulp and paper industry for various purposes, and for pharmaceutical applications (Chapin III, 2002). It is also useful in the recycling of cellulose and other polymers and biodegradable containers, thus minimising pollution (Victor, 1972), improving the digestibility and nutritional value of various novel food sources such as single cell protein (SCP), soybean products and as a treatment for phytobezoars (collections of indigestible cellulose material found in the gastrointestinal tract, usually the human stomach), etc. Nowadays moreover, cellulase is being used in the experimental fermentation of biomass into biofuels.

The invertebrate mollusc, African giant snail (*Archachatina marginata*), feeds on any edible plant or animal matter. These range from succulent fruits and vegetables e.g. paw-paw, banana and their leaves to ripe or overripe fruits, decaying organic material and grains (Wosu, 2001). The nature of their food will require an effective cellulase system for degradation and digestion. Some have reported possible endogenous enzyme sources are the hepatopancreas, gastric teeth and crystalline styles (needle-like structures made of crystalline proteins forming a motor organ in the stomach of bivalves) (Whitaker et al., 1963). In this work, we report the purification and characterization of a cellulolytic enzyme from the haemolymph of the African giant snail. The work arose out of curiosity when the Sephadex G-200 resin we were using to separate purported iso-enzymes of acid phosphatase (Afolayan and Agboola, 1996) was degraded by a fraction of the crude haemolymph.

MATERIALS AND METHODS

Snails were bought from the local market in Ile-Ife, Osun State, Nigeria. Bio-Gel P-300 was purchased from Bio-Rad Laboratories (Richmond, California, U.S.A.) while DEAE-Sephadex A-50 was from Pharmacia Fine Chemicals (Uppsalla, Sweden). CM-Cellulose (CMC), bovine serum albumin (BSA), Folin-Ciocalteu phenol reagent, Trizma base, Trizma-HCl, ovalbumin, catalase and o-nitrophenyl- β -D-glucopyranoside were obtained from Sigma Chemical Company Limited, St. Louis, Mo., U.S.A. All other reagents were of analytical grades and were obtained from reputable sources. Glass distilled water was used for all preparations of solutions and all pH measurements were made at 25°C using a Radiometer pH meter (Copenhagen).

Enzyme Purification

Crude extract

The snail's shell was carefully broken at the lower end to ensure the gut was not damaged. The haemolymph was allowed to drain into a beaker and filtered with a loose plug of glass wool.

Gel filtration on Bio-Gel P-300

A column (2.6 × 97 cm) of Bio-Gel P-300 was packed and equilibrated in accordance with Bio-Rad handbook in 10 mM sodium acetate pH 5.0 containing 1 mM EDTA. Then, 30 ml of the crude extract containing 1674.92 mg of protein was applied on the column. The protein was eluted with the equilibration buffer at a flow rate of 10 ml/h and then 5 ml fractions were collected. The fractions containing cellulase activity were pooled and concentrated with Amicon ultrafiltration PM 10 membrane.

First DEAE-Sephadex A-50 chromatography

DEAE-Sephadex A-50 was swollen and packed into a column (2.5 × 30 cm) according to Pharmacia Ion Exchange Chromatography Handbook (Ion Exchange Chromatography, Principles and Methods, 3rd Edition, 1991, Pharmacia LKB Biotechnology, Uppsalla, Sweden). The column was equilibrated with 50 mM ammonium acetate at pH 4.9. The concentrated sample of cellulase from the previous step was applied to the column. The column was eluted stepwise with 50, 100, 600 and 800 mM ammonium acetate (pH 4.9) and collected into 5 ml fractions. The fractions containing the highest cellulase activity were pooled and concentrated with Amicon ultrafiltration with pH 10 membrane.

Second DEAE-Sephadex Chromatography

The concentrated sample from the previous step was applied to another DEAE- Sephadex A-50 column (1.0 × 37 cm) which had been equilibrated with 50 mM Tris- HCl, pH 7.2. After washing the column with 40 ml of the same buffer, the enzyme was eluted with a stepwise gradient of NaCl (50, 100 and 150 mM) in 50 mM Tris-HCl (pH 7.2) at a flow rate of 7 ml/h. Fractions of 4 ml were collected. The fractions with cellulase activity were pooled and concentrated with Amicon ultrafiltration with pH 10 membrane.

Enzyme assays

CM-Cellulose assay

Cellulase activity toward CM-cellulose (CMCase) was measured by the appearance of reducing end groups in the solution of CMC. The assay mixture consisted of 0.5 ml of 1% (w/v) CMC (in 10 mM sodium acetate, pH 5.0 containing 1 mM EDTA) and 0.1 ml of the enzyme solution. This was incubated at 40°C for 30 min. The reducing equivalent was estimated with the Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952). A unit of cellulase activity was defined as the amount of enzyme that produces a change of an absorbance at 540 nm of 0.10 under the condition defined (Hurst et al., 1977).

Viscometric assay

The purified enzyme was assayed by monitoring the change in

viscosity in an Ostward viscometer. 13 ml of 0.6% CMC in 10 mM sodium acetate, at pH 5.0 containing 1 mM EDTA was mixed with 1 ml of the purified enzyme at room temperature. The activity was expressed as the percentage loss in viscosity of CMC according to Norkrans and Hammarstrom (1963).

β -Glucosidase assay

β -glucosidase activity was determined by the method of Umezurike (1976). The reaction mixture which was made up of 2.0 ml of 1 mM *o*-nitrophenyl- β -D- glucopyranoside (in 10 mM sodium acetate, pH 5.0 containing 1 mM EDTA) and 0.1 ml of enzyme was incubated for 30 min at 40°C. After incubation, 10 ml of 0.1 M sodium carbonate was added to 0.5 ml of the assay mixture. The nitrophenol released was measured by its absorbance at 400 nm. One unit of β -glucosidase activity was defined as that amount of enzyme needed to liberate 1.0 μ mole nitrophenol per minute under the conditions of the assay.

Protein concentration

Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard. During the purification, protein concentration was measured by taking the absorbance at 280 and 260 nm according to Warburg and Christian (1942) (Layne, 1957).

Polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gel electrophoresis (PAGE) under non-denaturing conditions was carried out on the purified enzyme to ascertain its purity, while PAGE in the presence of sodium dodecyl sulphate (SDS-PAGE) was performed for the determination of the subunit molecular weight and presence of carbohydrate. Non SDS-PAGE was performed on 7.7% separating gel and 4.6% stacking rod gel as described in the Pharmacia manual (Polyacrylamide Gel Electrophoresis, Laboratory Techniques, Revised Edition, Feb., 1983) using the Tris buffer system. SDS-PAGE was carried out according to the method of Weber and Osborn (1975) on a 10% Tris rod gel at pH 8.3. The gels were fixed for 12 h in 10% trichloroacetic acid (w/v) solution before staining. The standard protein mixture designated SDS-GH (Sigma product) was reconstituted in 1 ml of the sample buffer. The standard protein mixture contained myosin (M_r 205, 000), β -galactosidase (M_r 116,000), phosphorylase b (M_r 97, 000), bovine serum albumin (M_r 66, 000), egg albumin (M_r 45,000), and carbonic anhydrase (M_r 29, 000).

Molecular weight determination

The apparent molecular weight of the crude and purified cellulases were estimated by gel filtration on Bio-Gel P-300 column (2.6 \times 97 cm) which was calibrated with chymotrypsinogen A (M_r 25,000), ovalbumin (M_r 45,000), bovine serum albumin (M_r 66,000), γ -Globulin (M_r 160,000) and catalase (M_r 232,000) as marker proteins. The void volume (V_o) of the column was determined with haemocyanin.

Determination of kinetic parameters

The kinetic parameters (V_{max} and K_m) of cellulase were determined by varying the concentration of CMC from 0.33 to 0.83 mg/ml in 10 mM sodium acetate (pH 5.0) containing 1 mM EDTA. Data obtained

were analysed by the Lineweaver-Burk plot (Lineweaver and Burk, 1934).

Substrate specificity

Each substrate (concentration varies from 0.24 to 20 mg/ml) was incubated with the enzyme (10 μ g/ml) at 40°C in 10 mM sodium acetate (pH 5.0) containing 1 mM EDTA in a final volume of 1.0 ml. The incubation time ranged from 30 min to 24 h. The substrates included the plant glycosides (linamarin and salicin), the synthetic aryl β -glucoside (*o*-nitrophenyl- β -D-glucopyranoside) and the disaccharide (cellobiose), cotton wool and filter papers. The hydrolytic activity towards all substrates except *o*-nitrophenyl- β -D-glucopyranoside and cellobiose was measured by the appearance of reducing end groups with the Nelson-Somogyi method. The β -glucosidase assay had been described while cellobiose activity was determined as described by Hurst et al. (1977).

Effect of temperature on enzyme activity

An aliquot of the 100 μ g/ml purified enzyme in 10 mM sodium acetate (pH 5.0) containing 1 mM EDTA was incubated at the indicated temperature in a water bath for 15 min and allowed to cool in ice. The residual enzyme activity was determined according to the standard assay procedure.

Determination of carbohydrate content of the enzyme preparation

The total sugar content of the purified cellulase was determined according to the method of Dubois et al. (1956). The control experiment contained 4 mg/ml glucose. To determine whether the enzyme was a glycoprotein, SDS-PAGE was performed as earlier described. Gels were fixed for 12 h in 15% trichloroacetic acid to 25% isopropyl alcohol. The gels were then stained for carbohydrate using the periodic acid staining (PAS) procedure (Fairbanks et al., 1971) or as described by David (1979).

RESULTS

Purification

The results of the purification procedures are summarized in Table 1. The purification procedure yielded a homogenous cellulase with a specific activity of 1359 units/mg of protein. The fractionation of the crude extract by gel filtration on Bio Gel P-300 (Figure 1) was very effective in separating most other proteins from haemocyanin, which is a high molecular weight copper-containing haem protein that often forms complexes with other proteins. This separation method produced a major activity peak designated II with shoulders at peaks I and III which were all pooled together (Figure 1). The apparent molecular weights estimated from their elution volume were 19,000, 37,000 and 65,000 for I, II and III respectively. The fractionation of the post gel filtration sample on DEAE-Sephadex resulted in three activity peaks (designated AA, AB and AC) and a small one eluted at 600 mM ammonium acetate (pH 4.9) (Figure 2). The second ion exchange chromatography of AC yielded

Table 1. Summary of the purification of cellulose from the haemolymph of the giant African snail *A. marginata*.

Fraction	Volume (ml)	Total cellulose activity (Units)	Total protein (mg)	Specific activity (Unit/mg of protein)	Yield (%)	Purification fold
Haemolymph (Crude extract)	30	4755.0	1674.90	2.84	100.00	1.00
Gel filtration on Bio Gel P-300	78	2410.2	10.55	228.45	50.69	80.44
1 st DEAE-Sephadex A-50 Chromatography	32	428.6	1.74	244.60	8.95	80.13
2 nd DEAE-Sephadex A-50 Chromatography	26	598.0	0.44	1359.09	12.58	478.55

The enzyme was purified with a combination of gel filtration and ion-exchange chromatography. The samples were B, AC and ACB as designated in Figures 1 to 3 for gel filtration; 1st ion-exchange and 2nd ion-exchange chromatographic steps, respectively.

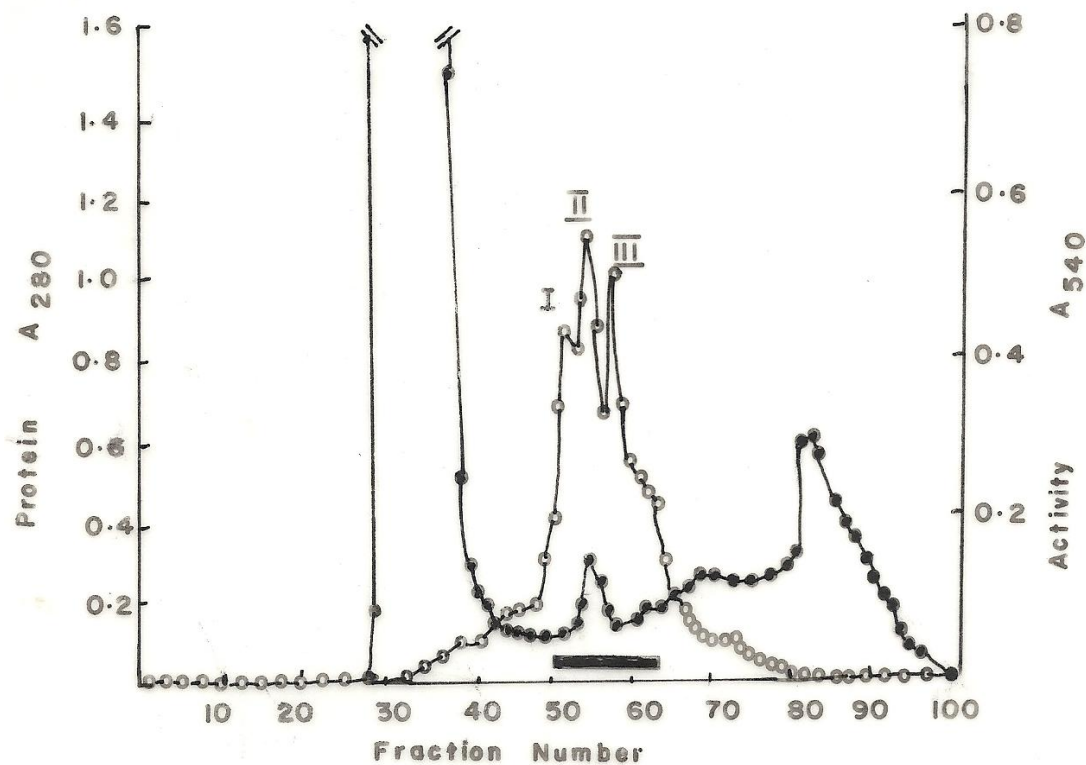


Figure 1. Elution of the crude extract (haemolymph) from a Bio Gel P-300 column. The crude extract (haemolymph) was applied to a (2.6 × 97 cm) column equilibrated with 10 mM sodium acetate pH 5.0 containing 1 mM EDTA. The flow rate was 10 ml/h and the fraction volume was 5 ml. The enzyme was eluted with the same buffer, and absorbance was read at A₂₈₀ (●-●-●) and A₅₄₀ (○-○-○). The solid bar indicates the pooled fraction.

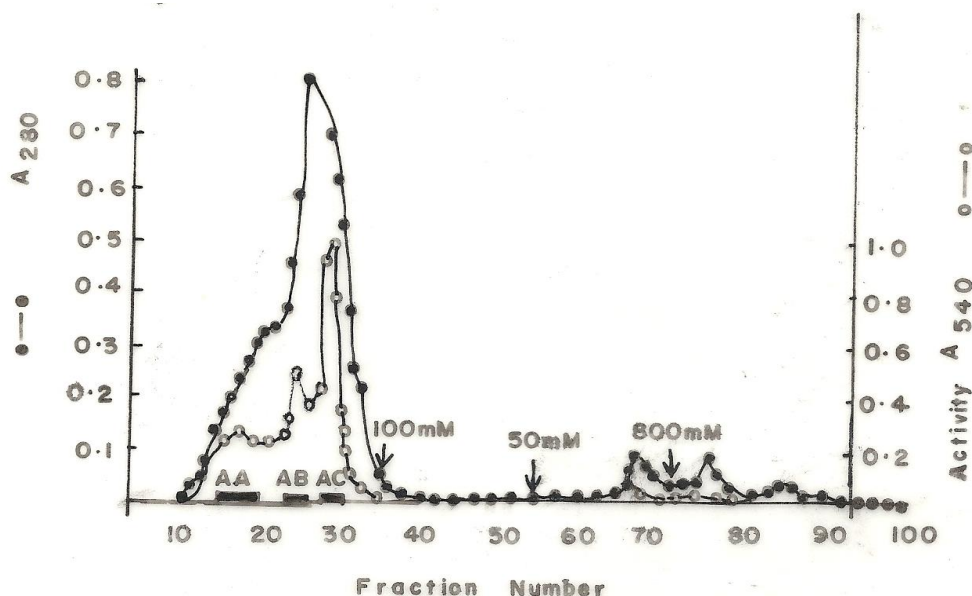


Figure 2. The elution profile of cellulase from DEAE-Sephadex A-50 column. The pooled fraction from the previous gel filtration step was first concentrated with amicon ultrafiltration cell with PM 10 membrane. The concentrated sample which contained 10.55 mg of protein with a specific activity of 228.45 units/mg was applied to a DEAE-Sephadex A-50 column (2.5 × 30 cm). The enzyme was eluted stepwise with 50, 100, 600, and 8800 mM ammonium acetate (pH 4.9) as indicated with arrowheads. The flow rate was 10 ml/h and fractions of 5 ml were collected and absorbance was read at A_{280} (●-●-●) and A_{540} (○-○-○). The solid bars indicate the pooled fractions.

two activity peaks; one of which was eluted with the starting buffer (ACA) and the other eluted with 150 mM NaCl (ACB) (Figure 3). The latter that was homogeneous (PAGE in the presence and absence of sodium dodecyl sulphate showed a single band of protein (Figure 4) was used for all characterisation experiments.

Molecular weights

The purified enzyme preparation has a native molecular weight of $52,000 \pm 2,050$ daltons as determined by gel filtration on Bio Gel P-300. The subunit molecular weight was estimated to be $16,875 \pm 1,556$ daltons by SDS-PAGE.

Kinetic parameters

The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of the purified cellulase for CMC were 5.17 ± 0.74 mg/ml and 1067 ± 195 units/ml respectively. The values obtained are the average \pm standard deviation from four determinations.

Substrate specificity

The result is presented in Table 2. The enzyme did not

degrade linamarin, salicin cellobiose, and o-nitrophenyl- β -D-glucopyranoside. In addition, its activity towards filter papers was very low compared with its activity towards CMC. Activities of the substrates were expressed as percentage CMC activity.

Effect of temperature on enzyme activity

The effect of temperature on the activity of the enzyme is presented in Figure 5. The activity was optimum at 30°C. At 50°C, the enzyme had lost half (56%) of its original activity. Little or no activity was detected at 60°C.

Viscometric assay

The effect of the cellulase on the viscosity of CMC solution is presented in Figure 6. Hydrolysis of CMC was accompanied by a rapid decrease in the viscosity of the solution. In the first 5 min, the viscosity of CMC had decreased by 52%, while at 15 min it had decrease by 80%, after which there was no more significant change in the viscosity.

Carbohydrate content

The total carbohydrate content of the enzyme preparation

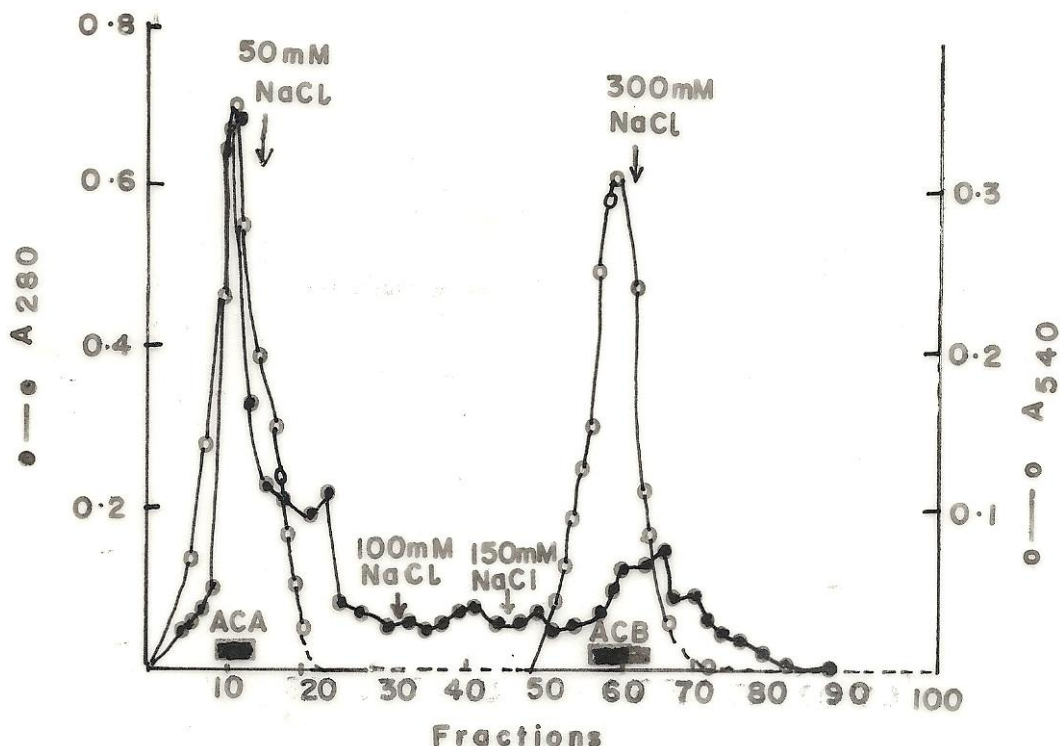


Figure 3. The elution profile of cellulase from second DEAE-Sephadex A-50 column. The fraction having the highest cellulase activity in the first DEAE-Sephadex A-50 were pooled and concentrated with Amicon ultrafiltration cell with PM 10 membrane. The concentrated sample which contained 1.74 mg of protein with a specific activity of 244.6 units/mg was applied to a DEAE - Sephadex A-50 column (1.0 x 37 cm). The enzyme was eluted with stepwise gradient of 0, 50, 100 and 150 mM NaCl in 50 mM Tris-HCl (pH 7.2) as indicated with arrowheads, the flow rate of which was 7 ml/h. Fractions of 4 ml were collected and absorbance was read at A_{280} (●-●-●) and A_{540} (○-○-○). The solid bars indicate the pooled fractions.

was 0.034 mg/ml. The staining for carbohydrate in the polyacrylamide gel by both the periodic acid staining (PAS) and thymol methods did not show any band of glycoprotein.

DISCUSSION

The overall result provides evidence for the existence of cellulase in the haemolymph of the giant African snail, *A. marginata*. No previous effort, to our knowledge, has been made to purify an endoglucanase or exocellobiohydrolase from the haemolymph of this tropical gastropod. This work also demonstrates the existence of more than one form of the enzyme in the haemolymph. The most prominent enzyme was purified to apparent homogeneity by a combination of gel filtration on Bio Gel P-300 and ion exchange chromatography on DEAE-Sephadex, and then characterized. This component could only degrade soluble forms of cellulose, CM-cellulose, with a high specific activity of 1359 units/mg of protein. More also, this cellulase from snail haemolymph could not hydrolyse linamarin (having a glucose unit and

2-hydroxyisobutyronitrile unit), salicin (having a glucose unit and benzoyl alcohol unit), *o*-nitrophenyl- β -D-glucopyranoside (having a glucose and nitrophenol units) and cellobiose. This therefore shows that the enzyme cannot accommodate changes in glucosyl residues to other monosaccharide or glucose derivatives.

The occurrence of a cellulolytic enzyme capable of hydrolysing soluble cellulose (CMCase) mainly may be because of the adaptation of the snail to majorly a vegetarian diet. Thus, it enables the animal to grow on the moist leafy vegetables, which form its major food sources. From the aforesaid, this fraction appears to be a C_x form of cellulase according to Olutiola and Ayres (1973) and Olutiola and Cole (1977). The reducing end equivalents released from substrates containing cellulose by the enzyme such as filter paper and cotton were very minimal, while glucopyranoside and cellobiose were not hydrolysed. Perhaps, β -glucosidase, which is that part of cellulolytic complex, is present in other fractions. It is noteworthy that earlier work in our laboratory had shown the presence of β -glucosidase in the haemolymph of snail, which is separable from the cellulase component by gel filtration on Bio Gel P-300 (Afolayan et al., 1997) and

Table 2. Substrate pecificity of cellulase from the haemolymph of the giant African snail (*Archachatina marginata*).

Substrate	Concentration of Substrate (mg/ml)	Linkage type	Incubation time (h)	Amount of enzyme in the reaction mixture (μ g)	Activity (% CM-Cellulase)
CM-Cellulase	10.00	β -1,4	0.5	10	100.00
Cellobiose	10.00	β -1,4	0.5	10	0.00
Linamarin	2.00	β -1,2	24	10	0.00
Salicin	10.00	β -1,6	24	10	0.00
o-Nitrophenyl- β -D-glucopyranoside	0.24	β -1,4	0.5	10	0.00
Filter paper (No 1)	20.00		24	10	0.20
Filter paper (No 2)	20.00		24	10	0.53
Filter paper (GP)	20.00		24	10	0.68
Cotton wool	10.00		24	10	3.76

Each substrate was incubated with 0.1 ml of the enzyme containing 10 μ g protein at 40°C for 30 min and 25°C for the 24 h incubation under the conditions defined. The assay was as used for CM-cellulose except for the substrate o-nitrophenyl- β -D-glucopyranoside, which was used to test for β -glucosidase activity of the cellulose.

not Sephadex G-200. It is also reasonable to suggest that the presence of β -glucosidase may be an adaptation of snails to diets containing plant glycosides. It is also interesting to note that we characterized a cyanide-detoxifying enzyme, rhodanese, in this snail for metabolising the toxic aglycone produced by the action of the glycosidase (Fagbohunka et al., 2004). The specific activity of 1359.09 units/mg of protein obtained for the enzyme was by far greater than the values obtained for endoglucanase from many fungi such as *Thermoascus aurantiacus*, which was 11.02×10^{-3} units/mg (Tong et al., 1981), *Aspergillus niger* which was 4.2×10^{-3} units/mg (Hurst et al., 1977) and *Clostridium thermicellum*, which was 65.1 units (Ng and Zeikus, 1981) that were all measured by the reducing sugar equivalent produced according to Nelson and Somogyi (Nelson, 1944).

The molecular weight of 52, 000 compares well with the values of 48,000 obtained for endoglucanase I from *Sclerotinia sclerotiorum* (Waksman, 1991), 49, 000 for endoglucanase II from *T. aurantiacus* (Tong et al., 1980), 51, 000

for the enzyme from *Trichoderma viride* (Hakansson et al., 1979) as determined by gel filtration. Values of 52, 000 and 49, 000 were obtained for endoglucanase III and IV, respectively from *T. viride* determined by sedimentation equilibrium technique (Shoemaker and Brown, 1978b). However, it is greater than 26, 000 obtained for cellulase from *Aspergillus niger* determined by SDS polyacrylamide gel electrophoresis and amino acid analysis (Hurst et al., 1977). It is also lower than 83,000 to 94,000 obtained for endoglucanase from *C. thermicellum* determined by ultracentrifugational analysis, amino acid composition and SDS polyacrylamide gel electrophoresis (Ng and Zeikus, 1981). The subunit molecular weight of 16, 874 obtained from sodium dodecyl sulphate gel electrophoresis suggest that the giant African snail haemolymph cellulase is at least a dimer. However, cellulase from other sources had been reported to be a single polypeptide protein (Hurst et al., 1977; Tong et al., 1980; Shepherd et al., 1981).

The K_m value of 5.17 mg/ml obtained for this enzyme is of the same magnitude with 3.9 mg/L

obtained for cellulase I from *Thermoascus aurantiacus* (Tong et al., 1980) and that of an endoglucanase from *S. sclerotiorum* (Waksman, 1991) which was 8.7 mg/ml. It is however higher than 0.5 mg/ml obtained for the cellulase of *Myrothecium verrucaria* (Halliwell, 1961). Moreover, Hurst et al. (1977) had reported a very high K_m of 52 to 80 mg/ml for the enzymes from *A. niger*. It has been shown that the K_m for CMC increases with an increase in the degree of substitution (Eriksson and Rzedowski, 1969). In this work, the CMC used has a degree of substitution of 0.7 to 0.8, while that of Tong et al. (1980) was 0.75. This probably explained the high K_m value of 19.0 mg/ml estimated by Stutzenberger (1971) for endocellulase hydrolyzing a CMC with a degree of substitution of approximately 1.2. Furthermore, the enzyme was stable up to 40°C, but was rapidly inactivated after incubation at temperatures above 50°C. Snails are poikilothermic whose physiological activities are dependent on the temperatures of the environment. Consequently, most of the enzymes in the snail including cellulase will be influenced

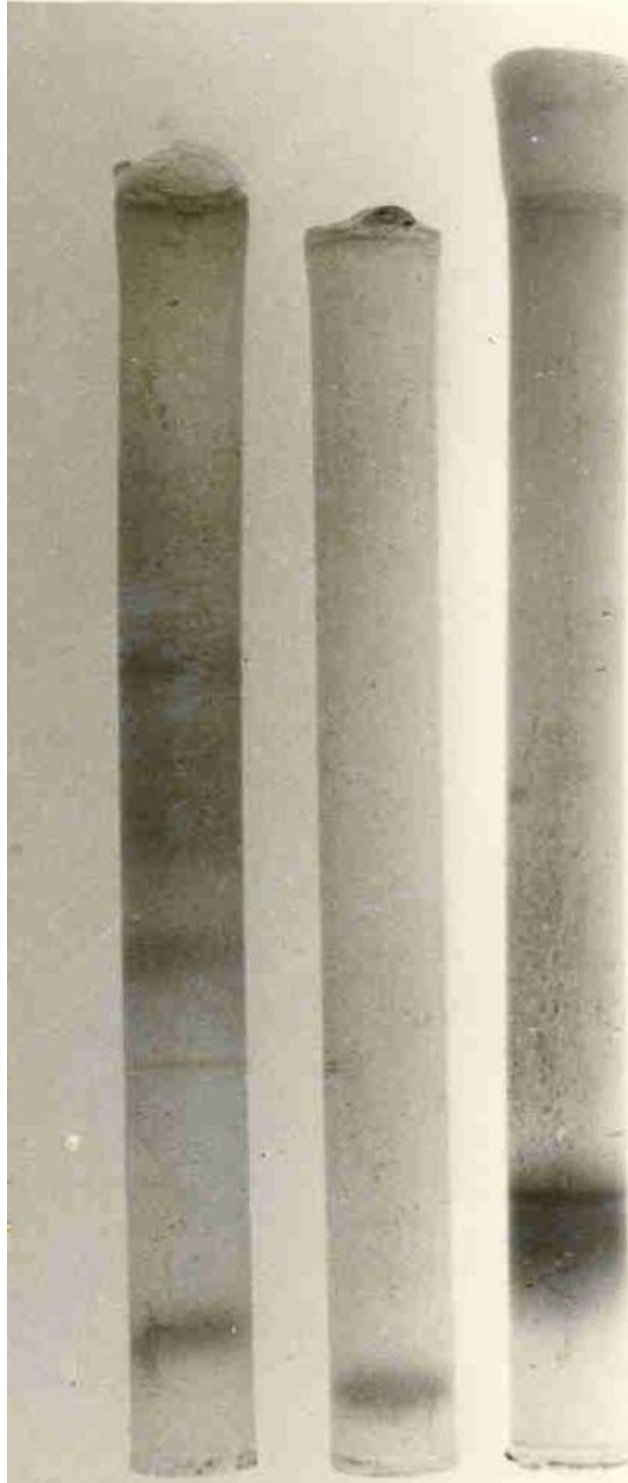


Figure 4. Photograph of the result of electrophoresis of cellulase after disc gel electrophoresis. Electrophoresis was in the absence of SDS (first and second from the left) and in the presence of SDS (third from left). The electrophoresis was carried out in 7.7% non SDS and 10% SDS-Tris gel. The first gel from the left is the non-SDS polyacrylamide gel of the partially purified enzyme harvested from the first DEAE-Sephadex A-50 column while the second and third are the non-SDS and SDS polyacrylamide gel of the pure enzyme respectively.

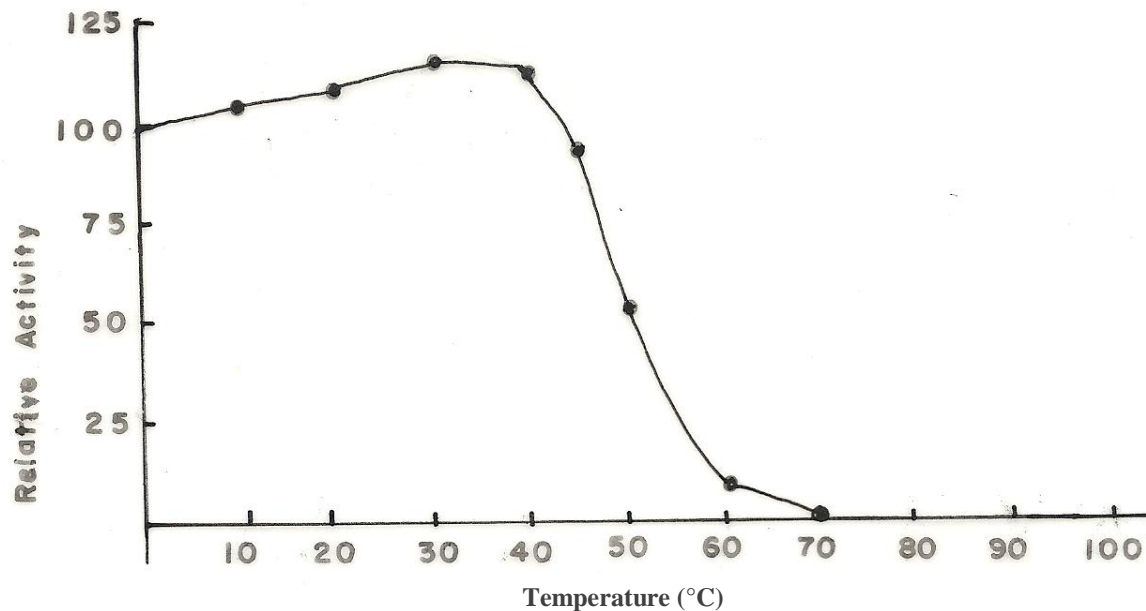


Figure 5. Effect of temperature on enzyme activity of snail cellulase. The percent residual activity was compared to the activity of the native enzyme at 0°C.

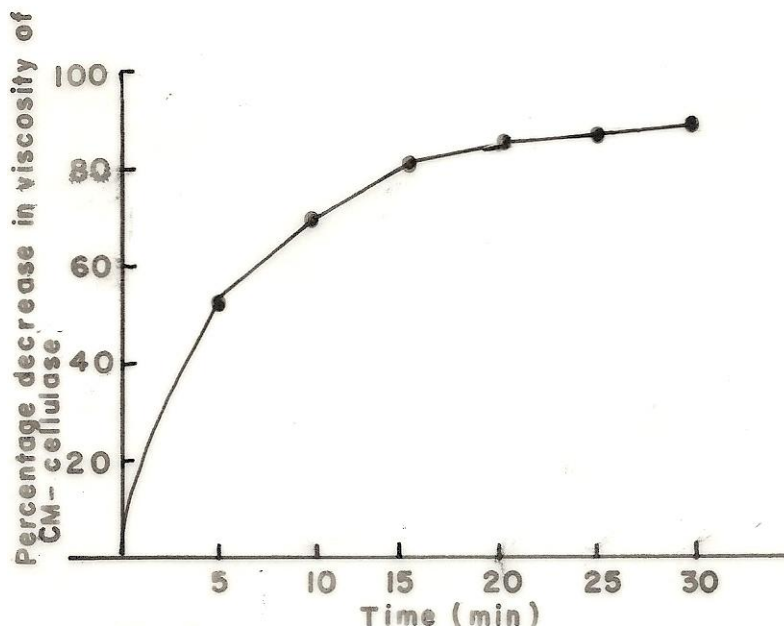


Figure 6. Decrease in viscosity of CM-cellulose by snail cellulase. A known volume (13 ml) of CM-Cellulose (6 mg/ml, in 10 mM sodium acetate pH 5.0 containing 1 mM EDTA) and cellulose (100 kg in 1 ml of the same buffer) was mixed thoroughly in an Ostwald viscometer at room temperature times. The efflux time of the mixture was taken at indicated times (the specific viscosity at zero time to the efflux time of the mixture for the first time immediately after mixing).

by the ambient temperature. Since the organism is never exposed to any ambient temperature higher than 40°C in the tropics, cellulase from *A. marginata* will probably be

more catalytically active *in vivo* throughout the wet and dry seasons of the year. This also explains the increase in activity at around atmospheric temperatures of 30 to

35°C. In contrast, it is characteristic of fungal cellulases to be highly thermostable up to 65°C (Hurst et al., 1977; Tong et al., 1980).

There was a rapid decrease in the viscosity of CMC (Figure 5). This is not different from the observations of Hurst et al. (1977), Shoemaker and Brown (1978a) and Tong et al. (1980). The rapid decrease in viscosity of CMC indicated that the enzyme has an endo- rather than exo-cellulolytic mode of action (Tong et al., 1980). Kanda et al. (1976) interpreted the magnitude of the slope as a measure of the "degree of randomness" of endoglucanases by assuming that the steeper the line, the more random the enzyme attack. Large positive slopes reflect the ability of an enzyme to cleave internal bonds in each of a number of cellulose molecules resulting in a large decrease in viscosity. Thus, multichain attack would be more likely to occur with a single (or few) bond cleavage(s) per enzyme substrate encounter (Hurst et al., 1977). Activity of enzymes (like exo-cellobiohydrolase) result in more nearly horizontal lines if represented by graphs which connote single chain attack with few or several bonds cleaved per encounter (Shoemaker and Brown, 1978a). Hence, for the fact that β -glucosidase had been separated from the sample, the high specificity for CMC and the rapid decrease in the viscosity of CMC, the enzyme in this work is likely to be an endoglucanase.

However, in contrast to endoglucanase from many fungal sources (Shoemaker and Brown, 1978; Tong et al., 1980), the enzyme has no detectable carbohydrate bound to it by the methods employed. Also, the enzymes from *A. niger* (Hurst et al., 1977) and *T. viride* (Hakansson et al., 1979) and even from bacteria (Osmundsvag and Goksoyr, 1975) and plant (Eryne et al., 1975) have been reported to be non-glycosylated. The fact that cellulase from the giant African snail is a non-glycoprotein can explain its low thermostability compared with those from fungal origin (Tong et al., 1980). Carbohydrates association with other proteins seems to confer heat stability on them (Jermyn, 1955). The value of carbohydrate content obtained in this work (0.034 mg/ml) might be from the DEAE-Sephadex A-50 resin digested by the enzyme. Digestion of DEAE-Sephadex A-50 was reported by Eriksson and Patterson (1968) in their purification of cellulase from *Sternum sanguinolentum*. Also, the snail haemolymph enzyme was found to digest Sephadex G-200 that was first used to fractionate acid phosphate and β -glucosidase from the haemolymph (Afolayan and Agboola, 1996; Afolayan et al., 1997).

REFERENCES

- Afolayan A, Agboola FK (1996). Presence of a singlet type of acid phosphatase in the haemolymph of giant African snail, *Archachatina marginata*. Nig. J. Biochem. Mol. Biol. 11: 57-63.
- Afolayan A, Agboola FK, Akinloye OA (1997). Some properties of β -glucosidase from the haemolymph of the giant African snail, *Archachatina marginata*. Nig. J. Biochem. Mol. Biol. 12:43-51.
- Bala M, Appelbaum L, Almogy G (November 2008). Unexpected cause of large bowel obstruction: colonic bezoar. Isr. Med. Assoc. J. 10 (11): 829-30.
- Chanda NB, Hirst L, Manners DJ (1957). A comparison of isolichenan and lichenan from icelandia moss (*Cetraria islandica*). J. Chem. Soc. pp. 1951-1958.
- Chapin III FS, Matson PA, Mooney HA (2002). Principles of Terrestrial Ecosystem Ecology. Springer-Verlag New York, NY.
- David R (1979). Glycoprotein detection in poly acrylamide gel with thymol and sulphuric acid, Analyt. Chem. USA, 99: 474-476
- Dubois M, Gilles K, Hamilton JK, Rebers PA, Smith F (1956). Colometric method for determination of sugar and related substrates, Analyt. Chem. USA, 28: 350-356.
- Eriksson KE, Rzedowski WI (1969). Extracellular enzyme system utilized by the fungus *Chrysosporium lignorum* for the breakdown of cellulose, Arch. Biochem. Biophys. USA, 129(2): 689-695.
- Eriksson KE, Petterson B (1968). Extracellular enzyme system utilized by the rot fungus *Sternum sanguinolentum* for the breakdown of cellulose: I Purification of the cellulase, Arch. Biochem. Biophys. USA, 142: 142-148.
- Eryne H, Christon NV, Verma DPS, Madachain GA (1975): Purification and characterization of two cellulases from Auxin treated Pea Epicotyls. J. Biol. Chem. USA, 250(8): 1012-1018.
- Fagbohunka BS, Adenuga GA, Okonji RE, Agboola FK (2004). Properties of Rhodanese (thiosulphate sulphur transferase) from the hepatopancreas of giant snail, *Archachatina marginata*. Sci. Focus, 7: 76-80.
- Fairbanks G, Steck LT, Wallach DFH (1971). Electrophoretic analysis of the major polypeptide of the human erythrocyte membrane. Biochem. USA, 10(13): 2606-2617.
- Hakansson U, Fagerstam LG, Petterson LG, Andersson L (1979). A 1-4- β -glucan glucanohydrolase from the cellulolytic fungus *Trichoderma viride* QM 9414, Biochem. J. Great Br. 179: 141-149.
- Hurst PL, Nielson J, Sullivan PA, Shepherd MG (1977). Purification and Properties of cellulase from *Aspergillus niger*. J. Biochem. 165: 33-41.
- Jermyn MA (1955). Fungal Cellulases IV. Production and Purification of an extracellular β -glucosidase of *Stachybotrys atra* Australian. J. Biol. Sci. Australia, 8: 541-562.
- Kanda T, Wakabayashi K, Nisizawa K (1976). Purification and properties of two cellulase components obtained from *Irpex lacteus* (*Polyporus tulipiferae*). J. Biochem. Tokyo, 79: 977-988.
- Layne E (1957). Spectrophotometric and turbidometric method for measuring proteins in methods in Enzymeol. Colowick SP, Koplman NO Eds; Academic press, New York, 3: 447-454.
- Lineweaver H, Burk D (1934). The determination of enzyme dissociating constants. J. Am. Chem. Soc. USA, 56:656-666.
- Lowry OH, Rosebrough NJ, Farri AL, Randal RJ (1951). Protein measurement with the folinphenol reagent, J. Biol. Chem. USA, 193: 265-275.
- Nelson N (1944). Photometric adaptation of Somogyi method for the determination of Glucose. J. Biochem. USA. 153: 375-380.
- Ng TK, Zeikus JG (1980). Purification and Characterization of an endoglucanase (1,4- β -D-glucanglucanohydrolase from *Clostridium thermocellum*. Biochem. J. Great Br. 199: 341-350.
- Norkrans B, Hammerstron A (1963). Studies on growth of *Rhizinia plantarum*, Fr. And its production of cellulose and pectin-decomposing enzymes physiological plantarum, Denmark, 16: 1-10.
- Olutiola PO, Cole OO (1976). Production of cellulase complex in culture filtrates of *Aspergillus tamari* associated with mouldy cocoa beans in Nigeria. Physiol. Plant. 36: 309-312.
- Olutiola PO, Ayres PG (1977). A cellulase complex in culture filtrates of *Rhychosporium secalis*, Trans. Br. Mycol. Soc., Great Britain, 60: 273-282.
- Osmundsvag K, Goksoyr J (1975). Cellulases from *Sporocytophaga myxococcoides*. Purification and Properties. Eur. J. Biochem. Germany, 57: 405-509.
- Peat S, Whelan J, Roberts JG (1957). The structure of lichenin. J. Chem. Soc. pp. 3916-3924.
- Pitiakoudis M, Tsaroucha A, Mimidis K et al., (Provide Complete Name) (2003). "Esophageal and small bowel obstruction by occupational bezoar: report of a case". BMC Gastroenterol. 3: p. 13.

- Shepherd MG, Tong CC, Cole AL (1981). Substrate specificity and mode of action of the cellulase from the thermophilic fungus *Thermoascus aurantiacus*. *Biochem. J. Great Britain*, 193: 67-74.
- Shoemaker SP, Brown Jr. RD (1987a). Enzymic activities of endo-1-4- β -D-glucanases purified from *Trichoderma viride*. *Biochem. et Biophysica Acta, Netherlands*, 523: 133-146.
- Shoemaker SP, Brown Jr. RD (1987b). Characterization of endo-1-4- β -D-glucanases purified from *Trichoderma viride*. *Biochem. et Biophysica Acta, Netherlands*, 523: 147-161.
- Somogyi MJ (1952). Notes on sugar determination. *J. Bio. Chem. USA*, 194: 19-24.
- Strutzenberger FJ (1971). Cellulase production by *Thermomonospora curvata* isolated from municipal solid waste compost. *Appl. Microbiol. USA*, 22(2): 147-152.
- Tong CC, Cole AL, Sheperd MG (1980). Purification and properties of the cellulases from the thermophilus fungus, *Thermomoascus auriantiacus*. *Biochem. J. Great Britain*, 191: 83-94.
- Umezurike GM (1976). The beta glucosidase in the gut content of the snail (*Archatina achatina*). *Biochem. J.* 157: 381-387.
- Waksman G (1991). Purification and Characterization of two endo-1:4- β -D- glucanses from *Sclerotinia sclerotiorum*, *Biochemical et Biohysica Acta, Netherland*, 1073:49-55.
- Warburg H, Christian W (1942). Isoleirung and Kristallization des garungsferments Enolase *Biochemische Zeitschrift, Germany*, 310: 384-421.
- Watanabe H, Noda H, Tokuda G (2001). Animal cellulases. *CMLS, Cell. Mol. Life Sci.* 58: 1176-1178.
- Whitaker DR, Hanson KR, Datta PK (1963). Improved procedures or preparation and characterization of *Myrothecium* cellulase. Part 2. Purification procedures. *Can. J. Biochem. Physiol.* 41: 671-696.
- Woodward J (1995). Paladium-a new inhibitor of cellulase activities. *Biochem. Biophys. Res. Commun.* 209: 1046-1052.
- Woodward J, Stephen Koran L, Idong K, Saddler J, Saddler Y (1994). Enzymatic separation of higher quality unliked pulp fibres recycled newspapers. *Biotechnology*, 12: 905-908.