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α,β-Glucan Complex in *Saccharomyces carlsbergenesis*

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The two pools of glycogen present in *Saccharomyces carlsbergenesis* one is the soluble pool and intracellular, the remainder water insoluble linked with cell wall β-glucan. Glycogen is universally assigned the role of an energy reservoir and is always present intracellularly. The intracellular pool of glycogen which could be broken down to yield glucose and further used for production of energy required for metabolic activities. The soluble pool of glycogen present in the *Saccharomyces carlsbergenesis* could have performed this function efficiently; however the presence of an alternate pool in the cell wall would indicate that its physiological role should be different from that of the soluble glycogen. It is demonstrated that this glycogen can be synthesized using the alkali insoluble cell wall pellet as a primer. The attachment of cell wall glycogen to β-glucan was established in laboratory through the use of (1→3)-β-D-glucanase, β-glucosidase and lyticase enzymes, used for solubilization of cell wall linked glycogen, concanavalin A sepharose CL-4B affinity chromatography and molecular sieving has confirmed that glycogen is linked to β-glucan. Further partial acid hydrolysis and subsequent paper chromatography of the α,β-glucan complex revealed the presence of gentiobiose and other higher oligosaccharide. This proved that glycogen is linked to β(1→3) through β(1→6) branch in *Saccharomyces carlsbergenesis*.

Key Words: *Saccharomyces carlsbergenesis*, α,β-Glucan, Yeast glycogen, Two pools of glycogen.

INTRODUCTION

The polysaccharide glycogen, a branched polymer of glucose is widely distributed in nature and acts as a storage molecule accumulated in times of nutritional sufficiency, for later utilization under conditions of starvation. Glycogen has been isolated and characterized from a variety of micro-organisms including yeasts, *Mycobacteria*[1], *E. coli*[2] and *Aerobactor aerogenes*[3]. Furthermore, precise information about the structure of yeast glycogen was given by Northcote[4].

Cell wall of yeast made up of glucan, mannan and chitin *etc*. The gross chemical content of the isolated baker's yeast cell wall is 83 % carbohydrate, 10 % protein, 3 % lipid, 0.45 % sterol, 0.3 % ribonucleic acid and

0.04 % deoxyribonucleic acid[5]. β-Glucan is the insoluble component present in yeast cell wall contains β(1→3), β(1→6) and β(1→4)linkages. The major alkali insoluble component or alkali stable component[6] contains predominantly β(1→3) linkages with few β(1→6) and β(1→4)linkages while a minor component which is acid soluble contains a major fraction rich in β(1→6) linked glucan[7] with few β(1→3) linked glucose residues[8]. Mannan is made up of mannose residues and it is a glycoprotein[9] and chitin is mostly present in bud scars[10].

Besides these well accepted components of the cell wall, there is a presence of glycogen in the periplasmic space[11]. A report showing the alkali insoluble fraction being associated with one pool of glycogen indicates that glycogen in yeast may exist in two pools. One intracellularly while other cell wall bound[12]. The solubilization of insoluble glycogen fraction in *Saccharomyces cerevisiae* using (1→3)-β-D-glucanase, it's purification by affinity chromatography using concanavalin-A sepharose CL-4B, molecular sieving and partial acid hydrolysis indicating that glycogen is linked to β(1→3) glucan through a β(1→6) branch[13].

The present study is carried out to establish the presence of glycogen in the *Saccharomyces carlsbergenesis* and to predict the nature of the component to which the glycogen is linked.

EXPERIMENTAL

Sepharose CL-4B concanavalin-A, amyloglucosidase from *Aspergillus niger*, lyticase from *Arthobacter luteus* and laminarin purchased from Sigma Chemicals USA. Biogel P-2 were purchased from BioRad Lab, Richmoan, Cal USA.

Organisms and culture conditions: *Saccharomyces carlsbergenesis* 3312 was purchased from National Chemical Laboratory, Pune were maintained on agar slants having the composition 1 % glucose, 0.5 % peptone, 0.3 % yeast extract and 2.5 % agar. For a detail chemical and enzymatic analysis of insoluble glycogen pool, the bulk amount of cells which were grown in 10 % sucrose, 0.5 % peptone and 0.3 % yeast extract. The fermentation carried at room temperature (25 ± 2 ºC) and cells were harvested after 48 h, centrifuged under cold conditions at 6000 rpm for 10 min, washed repeatedly with cold water and analyzed further.

Alkali digestion of yeast: The alkali digestion of cells was done by using 2 mL of 20 % KOH/g of yeast cells and were kept in a boiling water bath for 1 h, cooled in an ice bath and the pH of the alkali digested yeast cells was adjusted to pH 7 by using ice cold 0.5 M HCl. The digest was centrifuged. The supernatant obtained contained soluble glycogen and other components. The insoluble jelly like mass that separates out was washed with distilled water till the supernatant yields negative test with phenol

sulphuric acid. The insoluble mass obtained contained mostly β-glucans of cell wall to which α-glucans are linked.

Estimation of soluble and insoluble glycogen: The glycogen was estimated specifically using amyloglucosidase and α-amylase mixture. The liberated glucose was assayed using glucose oxidase-peroxidase method. The amyloglucosidase (*Aspergillus niger*) used for estimation was prepared by dispersing 35 mgs of the lyophilized powder of amyloglucosidase in 10 mL of sodium acetate buffer (100 mM, pH 4.2) centrifuged and used for the treatment. The salivary α-amylase was prepared in the laboratory. Mixture of amyloglucosidase and α-amylase were used for the degradation of the glycogen. For routine estimation 0.5 mL of substrate was taken, treated with enzyme for 1 h at 37 °C. The reaction was terminated by placing the tubes in a boiling water bath for 3 min. After cooling, the reaction mixture was centrifuged and liberated glucose was then estimated by specific glucose oxidase peroxidase method.

Treatment of (1→3)-β-D-glucanase, β-glucosidase and lyticase to insoluble mass: The (1→3)-β-D-glucanase and β-glucosidase from *Penicillium ochoro-chloron*[14] were used for the treatment of insoluble mass. The alkali insoluble mass suspended in citrate buffer pH 5 (5 mL) and 0.5 mL of (1→3)-β-D-glucanase or β-glucosidase enzyme was added. The suspension was incubated for 5 h at 50 °C and centrifuged

Lyticase obtained from *Arthobacter luteus* was also used for the treatment of insoluble mass to isolate α-glucans linked to β-glucans of the cell wall. The enzyme has protease and (1→3)-β-glucanase activity. The β-glucanase activity was tested on the commercially purchased laminarin. The alkali insoluble mass was suspended in potassium phosphate buffer (5 mL) and 0.5 mL of the lyticase enzyme was added. The suspension was incubated for 1 h and centrifuged.

The repeated enzyme treatment was needed for all enzymes to obtain a near complete isolation of α,β-glucan complex. The supernatant and insoluble mass were tested after every treatment with iodine qualitatively to test the extent of release of α-glucan with a proper control. The supernatants were pooled together, dialyzed and analyzed for α-glucans, total carbohydrate, lyophilized and stored for further analysis. The treatment of (1→3)-β-D-glucanase, β-glucosidase and lyticase preparations indicated more or less similar results.

Concanavalin-A-sepharose CL-4B affinity column chromatography: Concanavalin-A immobilized on sepharose CL-4B was routinely used. A column of length 6 mL was run at +4 °C in a glass syringe. The flow rate was adjusted to 5 mL/h. The column was operated[15,16] in *tris* buffer 10 mM containing 1 mM each of $CaCl_2$, $MgCl_2$, $MnCl_2$. The carbohydrate fraction which was obtained after lyticase or (1→3)-β-D glucanase

or β-glucosidase were loaded on this column separately. The nonretained fractions eluted were mostly β-glucans. The column was then washed with the same buffer until free from the non-retained fraction. The retained fraction was eluted with the same buffer containing 60 mg/mL of α-D-glucose solution. The held α-glucan fraction eluted was tested with iodine solution. The fractions containing α-glucans were pooled together dialyzed tested for total carbohydrate and α-glucan content and subjected to enzymatic analysis.

Biogel P-2 gelfilteration chromatography: The fraction which is purified by concanavalin-A sepharose CL-4B column chromatography was further analyzed by using biogel P-2 column chromatography. The same fraction was treated with amyloglucosidase and the products obtained were also analyzed with biogel P-2 column chromatography length 90 cm and diameter 1.28 cm in potassium phosphate buffer (pH 7.0, 10 mM).

Acid hydrolysis of α-β-glucan complex: The α,β-glucan complex obtained from biogel P-2 column, retarded peak of Fig. 3 was subjected to partial acid hydrolysis. About 1mg of the α,β-glucan complex was hydrolyzed with 1 M H_2SO_4 for 1 h hydrolysate was neutralized using solid barium carbonate and the supernatant analyzed by descending paper chromatography using solvent system butanol:ethanol:water (4:1:5) The reaction mixture was spotted with glucose, maltose and gentiobiose as standards. The separated sugars were visualized using spray reagents like alcoholic sodium hydroxide and acetonic silver nitrate.

Determination of specific optical rotation: The solution of 5 % sucrose prepared in distilled water was used as a standard. 0.1 M NaOH was used as a solvent for partially insoluble α-β-glucan complex instead of water and a blank reading was recorded using 0.1 M NaOH.

RESULTS AND DISCUSSION

In yeast, two pools of glycogen were present, one pool is cytoplasmic in origin and the other is bound to the cell wall. In view of all these facts, the present study was undertaken. A detailed chemical and enzymatic analysis has been carried out to establish the presence of this pool of glycogen in *S. carlsbergenesis*. The cells harvested from the nutrient medium are subjected to alkali digestion and then total soluble and insoluble carbohydrate is analyzed[17]. The soluble and insoluble α-glucan contents were measured using specific glucose oxidase and peroxidase method.

Table-1 shows the content of soluble, insoluble carbohydrate and soluble, insoluble glycogen, which is routinely, obtained when cells grown in 10 % sucrose, 0.5 % peptone and 0.3 % yeast extract. The glycogen is expected to be linked with the cell wall β-glucan and it should be solubilized by the use of a β-glucanases. In the present study, (1→3)-β-D-glucanase and β-glucosidase from *Penicillium ochro-chloron* and lyticase,

a preparation form *Arthobacter luteus* containing protease and β(1→3) glucanase activity is used for the treatment of insoluble mass. Table-2 includes the data of amount of α-glucans released from the insoluble mass after each treatment. The insoluble mass containing 115 mg of α-glucans required at least 4 enzyme treatments for complete isolation from insoluble mass. The isolated carbohydrate was collected and lyophilized. The lyticase preparation from *Arthobacter luteus* had endoglucanase activity and the random action of the enzyme on insoluble mass released free β-glucans in addition to α,β-glucan complex.

TABLE-1
CONTENTS OF SOLUBLE AND INSOLUBLE TOTAL CARBOHYDRATE AND GLYCOGEN

Carbohydrate	Soluble (mg/g of yeast cells)	Insoluble (mg/g of yeast cells)
Total carbohydrate	42.0	58
α-Glucans	5.1	15

TABLE-2
EXTENT OF RELEASE OF α-GLUCANS AFTER LYTICASE TREATMENT

Lyticase action	Insoluble glycogen (mg)
I treatment	6.6
II treatment	9.8
III treatment	42.4
IV treatment	40.5

When the (1→3)-β-D-glucanase from *Penicillium ochro-chloron* was used for the treatment of insoluble mass, it was observed that repeated treatment was necessary for a near complete isolation of the glycogen. The isolated carbohydrate was collected, dialyzed and lyophilized. The enzyme (1→3)-β-D-glucanase showed endoglucanase activity and the random action of the enzyme on insoluble mass also releases free β-glucans in addition to α-glucan β-glucan complex. The isolated mass was less turbid as compared to the isolated mass after lyticase treatment. Similarly, the β-glucosidase was also used for treatment of insoluble mass to isolate α-glucan β-glucan complex and was further analyzed.

Purification of α-β-glucan complex by affinity chromatography: It was noted that (1→3)-β-D-glucanase or β-glucosidase or lyticase treatment on the insoluble mass releases not only α,β-glucan complex but also some free β-glucans and separation of these two homopolymers having

the same sugar units was found to be essential. The separation of these two homopolymers by conventional method was not easier and hence warranted a specific technique which can separate the two homopolymers. The advent of lectin and their selective specificity for sugars provided a valuable tool for this purpose. Concanavalin-A lectin has affinity towards α-glucans and its lack for β-glucans makes it an ideal choice for this purpose. The fraction released after the treatment of lyticase to insoluble mass when loaded on the column of immobilized concanavalin-A, the β-glucans present in the complex eluted out as a non-retained fraction. Elution profile of the concanavalin-A affinity column chromatography is depicted in Fig. 1.

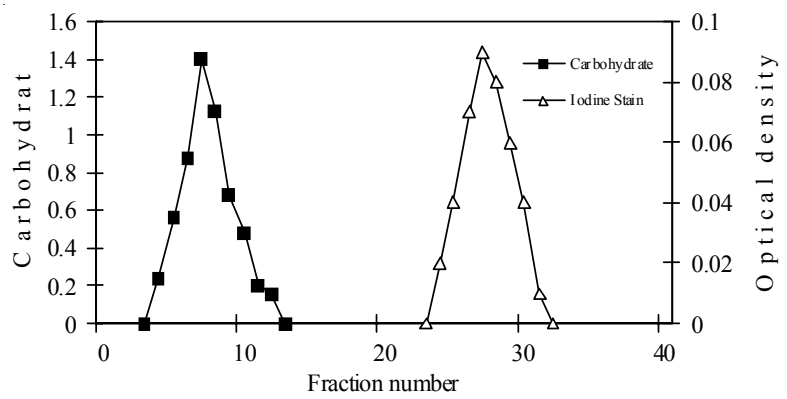


Fig. 1. Elution profile of solubilized glucan complex obtained by alkali digestion and lyticase treatment

It is obvious that concanavalin-A has no affinity for peak 1 of the Fig. 1. The retarded fraction was selectively eluted out by the addition of glucose in the same buffer. When 25 mg of carbohydrate loaded on this column, 7 mg eluted out as the non-retained fraction while about 19.8 mg eluted out as the retained α-glucan rich fraction. The elution of retarded fraction was monitored by iodine stain method. The eluted fraction was collected and dialyzed exhaustively to remove glucose, used for elution. The fraction was lyophilized and used for further analysis. Similarly, the α,β-glucan component released after treatment of (1→3)-β-D-glucanase and β-glucosidase were purified

Analysis of fraction after (1→3)-β-D-glucanase treatment on Biogel P-2: The α,β-glucan complex which releases after (1→3)-β-D-glucanase treatment was purified by concanavalin-A chromatography and analyzed further on biogel P-2 column. Fig. 2 shows the elution profile. The fraction immediately eluted out as non-retained fraction towards the end of the void volume. The void volume of column was determined by passing blue

dextran and found to be 38 mL. The elution of the non-retained fraction began by the 38th fraction and it eluted out as a single peak. The non-retained fraction from Fig. 2 was pooled and treated with amyloglucosidase and α-amylase and reloaded on the same calibrated column. The peak which was eluting out as a non-retained fraction towards the end of the void volume now elutes into two peaks one between 44th and 49th fraction and the other between 85th and 90th fraction. Elution profile is shown in Fig. 3. The observed shift in the peak 1 in Fig. 2 as compared to that of the retarded peak in Fig. 3 signified that the high molecular weight α-glucan and β-glucan on treatment with amyloglucosidase degrades and releases glucose which separates as peak 2 while the retarded peak in Fig. 3 indicates that the presence of small β-glucan chain which was linked to the high molecular α-glucan component. No protein was detected in this fraction. A controlled acid hydrolysis of this fraction, was carried out further for linkage studies. Since, the content of the peak 1 in Fig. 3 was noted to be very less; this fraction was collected in series of runs. These were thought to be residual β-glucan linked to α-glucan structures.

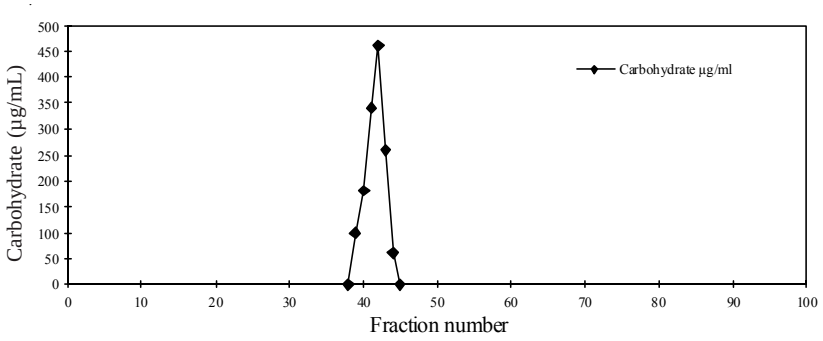


Fig. 2. Eluion profile of solubilized glucan complex by (1:3) β-D-glucanase treatment

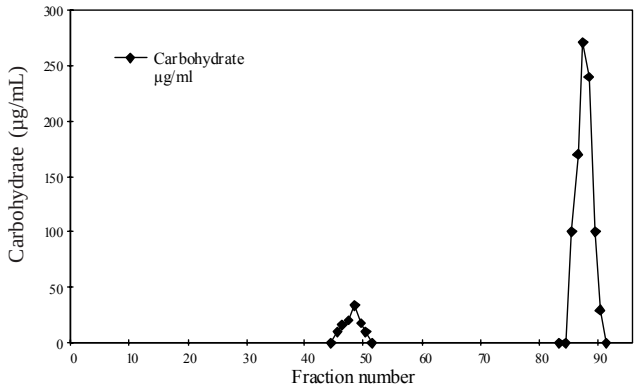


Fig. 3. Elution profile of non-retained fraction from fig. 2 amyloglucosidase treatment

Analysis of fraction after β-glucosidase treatment on Biogel P-2: The α-glucan β-glucan complex which is released after β-glucosidase treatment was purified by concanavalin-A chromatography and was analyzed further on biogel P-2 column. Fig. 4 shows the elution profile. The calculated void volume is 38 mL and the loaded fraction immediately eluted out as non-retained fraction towards the end of the void volume. The elution of the non-retained fraction began by the 38th fraction and it eluted out as a single peak. The non-retained fraction from Fig. 4 was pooled and treated with amyloglucosidase and α-amylase and reloaded on the same calibrated column. The elution profile observed is shown in Fig. 5. The peak which was eluting out as a non-retained fraction towards the end of the void volume elutes into two peaks, one between 50th and 53rd fraction and the other between 85th and 90th fraction. From these observations, the authors

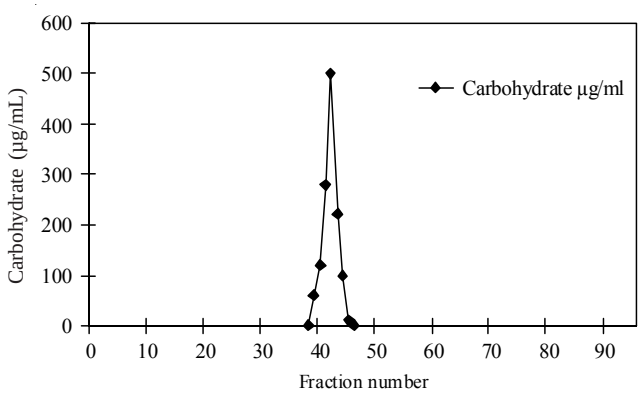


Fig. 4. Elution profile of solubilized glucan complex by β-glucosidase treatment

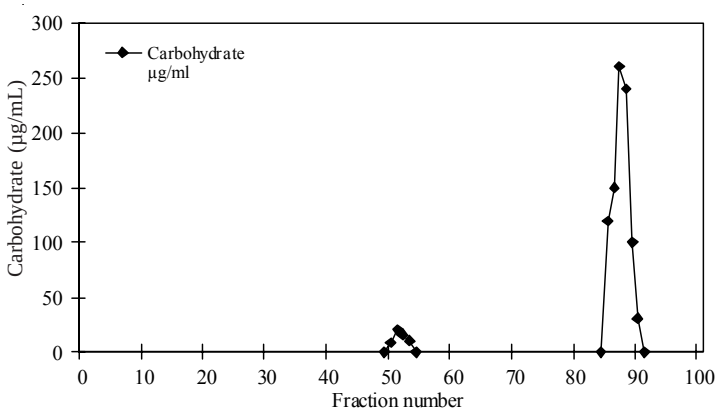


Fig. 5. Elution profile of non-retained fraction from fig. 4 after amyloglucosidase treatment

draw a conclusion that the peak which is eluted as non-retained fraction belongs to the high molecular weight α,β-glucan on treatment with amyloglucosidase and α-amylase degrades and releases glucose which separates as peak 2 and the retarded peak in Fig. 5 as the small β-glucan chain which was linked to the high molecular α-glucan component.

Simillarly α-glucan β-glucan complex which gets released after lyticase treatment was purified by concanavalin-A chromatography and was analyzed further on Biogel P-2 column shows similar elution pattern as that of (1→3)-β-D-glucanase and β-glucosidase (data not shown).

Thus fractions obtained after (1→3)-β-D-glucanase treatment and β-glucosidase treatment when purified by using concanavalin-A and passed through biogel P-2 column gets eluted out as a non-retained fraction at 38th fraction. However, when these fractions were collected and treated with amyloglucosidase and α-amylase, the (1→3)-β-D-glucanase treated fraction elutes between 44th and 49th fractions. The β-glucosidase treated fraction eluted between 50th to 53rd fractions indicates that β-glucosidase is a non specific enzyme. It cleaves some of the β linkages from the α,β-glucan complex and reduces its molecular weight and hence get eluted in further fractions. These observations clearly prove that α-glucans are covalently linked to β-glucans in the yeast cell wall. The peak 1 obtained in Fig. 3 and 5 was found to contain only glucose. Therefore, it is certain that the synthesis of this pool of glycogen does not need a protein as a primer. In case of mammalian glycogen glycogenin acts as a primer for the action of glycogen synthetase to proceed and synthesize glycogen[18]. It is evident that in the yeast *Saccharomyces carlsbergenesis* the carbohydrate component should serve as a primer. The linkage of α-glucan to the β-glucan was studied further by making the experiments of acid hydrolysis.

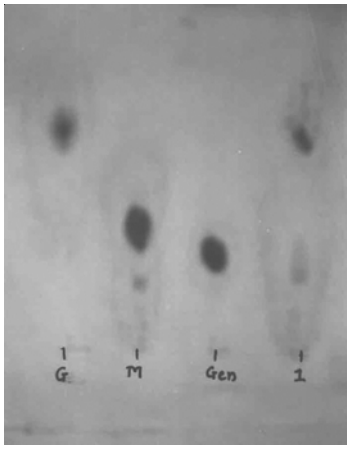
The partial acid hydrolysis of the α β glucan complex obtained by (1→3)-β-D-glucanase treatment revealed the presence of gentiobiose and higher oligosaccharides. Thus it appears that glycogen in yeast is linked to β (1→3) glucan through a β(1→6) branch (Photograph No.1).

Determination of specific optical rotation: The carbohydrate fractions, obtained after (1→3)-β-D-glucanase, β-glucosidase and lyticase treatment to insoluble mass and also from concanvalin-A sepharose column, were subjected to polar metric analysis to study the nature of this fraction. The specific optical rotation values of the various fractions obtained are presented in Table-3. The fraction which was isolated by action of lyticase to insoluble mass shows, optical rotation of +101°, while peak 1 of concanavalin-A column that is non-retained fraction which contain β-glucan shows a value of -35° optical rotation. The fractions which were obtain after (1→3)-β-D-glucanase and β-glucosidase treatment were less turbid as compared to fraction of lyticase treatment and exhibit the specific

rotation values of +115° and +121°, respectively, indicates that (1→3)-β-D-glucanase and β-glucosidase releases less amount of β-glucans in the reaction mixture. The purified glycogen shows 188-190° optical rotation but the peak 2 of concanavalin-A column that is a retarded fraction shows +156.5° optical rotation which is less as compared to pure glycogen, signifying that this peak contain β glucan attached to α-glucan, which is responsible for the decrease in optical rotation.

TABLE-3
OPTICAL ROTATION SHOWN BY VARIOUS CARBOHYDRATE FRACTIONS

Carbohydrate fraction	Optical rotation $[\alpha]_D^{25}$
Sucrose	+66°
Fraction after lyticase treatment to insoluble mass	+101°
Fraction after (1→3)-β-D-glucanase treatment to insoluble mass	+115°
Fraction after β-glucosidase treatment to insoluble mass	+121°
Peak 1 of concanavalin-A column. (non retained fraction)	- 35°
Peak 2 of concanavalin-A column (retained fraction)	+156.5°



G M Gen 1

Photograph No. 1: Paper chromatographic analysis of acid hydrolysis of α-β-glucan complex; G-Glucose, M - Maltose, Gen - Gentiobiose, 1- Reaction products of acid hydrolysis

The value reported earlier for α,β-glucan complex is +113° and the value observed in present studies after lyticase treatment is +101° but this value gets altered to +115° and +121° after the (1→3)-β-D-glucanase and β-glucosidase treatment, respectively. The variations observed may be due to the different experimental methods used for solubilization of the

α,β-glucan complex and hence the total optical rotation value obtained would be the sum of the different components present. The separation of β-glucans from α,β-glucan complex increases the value to +156.5° as observed in case of peak 2 of concanavalin-A column.

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