Enzymatic Modification of Locust Bean and Guar Gums

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The modification of locust bean gum and guar gum polymers was carried out by the specific hydrolysis of galactose from these polymers with a crude enzyme preparation. The crude enzyme was perpared from the extract of sprouted guar seed (sprout length 3-4 cm) by fractional precipitation with tannic acid. Although the crude enzyme was found to be a mixture of etght proteins, its specificity for alpha (1→6) linkage was remarkably good. Maximum enzyme activity was noticed at 38°C and pH 4.6 to 5.0. Effects of parameters such as time, pH, temperature, enzyme concentraction, and substrata concentration were studied.

The gums were characterized by the total acid-hydrolysis method. The locust bean gum was found to contain galactose and mannose whereas the guar gum showed the presence of galactose, mannose, glucose and arabinose.

The enzymatic hydrolysis of gums after 32 hours liberated over 60% of galactose. Further hydrolysis of guar gum (152 hr) liberated over 95% of galactose, 98% of arabinose and only 2.6% of mannose. Mannose was cleaved only on prolonged reaction periods and glucose was not attacked. During the course of hydrolysis a white precipitate was obtained in both cases which contained mainly mannose. The precipitation of mannan indicated that the mannopyranospl chain was practically linear.

Introduction.

Beta-galactosidases from various origins have been explored extensively but alpha-galactosidases have received relatively little study. The latter enzyme has been reported in yeast, filamentous fungi, plant seeds, bacteria, and in the digestive tract of some animals *(1–9)* Helfrich and Vorsatz *(10)* isolated the crude enzyme from the coffee bean extract by precipitation with the help of tannin, Suzuki, *et al. (11)* prepared the enzyme by incubating *Penicillium casei* on a sterilized mdium containing 3 g soyabean powder, 2g rice hulls, and 2.5 ml water. One of the characteristics of alpha-galactosidases which have been studied is their transferring activity. French *(12)* prepared a triose containing galactose and melibiose with alpha—galactosidase. The formation of triose is explained by the transfer of *D*-galactose moiety of melibiose. Transferring activity of alpha-galactosidasd has also been reported by Courtois, *et al. (13)*, wohnlich *(14)*, and Malhotra and Dey *(15)*. Lechevallier *(16)* while studying the evolution of alpha-galactosidase activity in the course of germination and maturation of various seeds found that all seeds contained alpha-galactosidase and transferase irrespective of their content of galactose-containing oligosaccharides. Clancy and Whelan *(17)* reported having polymerized galactose to the extent of 7.5% with the help of alpha-galactosidase. Heyne and Whistler *(18)* observed that a commercial diastase preparation caused a reduction in the viscosity of guaran solution. Courtois and his associates *(19)* used alpha-galactosidase from coffee beans to cleave galactose units selectively from a galactomannan.

Because of the specificity of the enzymes, enzymatic hydrolysis was regarded as the best tool for the selective hydrolysis of locust bean and guar gum galactomannans which are known to consist of a linear chain of *D*-mannose units

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linked together by β-(1→4) glycosidic linkages and having on certain *D* mannose units a single *D* galactose unit joined by an α-(1→6) glycosidic linkage*(20,21)*. The present work was undertaken to isolate an enzyme capable of specific hydrolysis af galactose from the locust bean and guar gum polymers in order to study the effects of the polymer mannan consent on the effectiveness of the modfied gums as beater adhesives in the paper industry.

Results and Discussion.

Analysis of the purified locust bean gum (Lp_1) and guar gum (GP_1) was carried out by the method of Saeman, *et al* (*22*). The gums were first acid-hydolyzed and the monomer sugars were quantitatively estimated by the micro-chromatographic method.

Upon hydrolysis, LP_1 gave 25.9 g galactose and 86.5 g mannose, whereas GP_1 gave 44.5 g galactose, 66.6 g mannose, 0.95 g glucose, and 1.13 g arabinose per 100 g dry basis of the respective polymers.

The initial assay of the enzymatic activity was carried out by comparing the efflux time of the locust bean gum solution (0.1%), before and after hydrolysis, in an Ostwald Viscometer. Qualitative checks for galactose and mannose were also made by paper chromatography.

The crude preparation obtained from the molds (see Experimental Section) showed very little changes in the efflux time and did not liberate any galactose or mannose from the gum. The enzyme preparation from the molds was, therefore, discarded. The crude enzyme obtained from germinated guar seeds, on the other hand, showed mainly alpha-galactosidase activity. The yield of this crude enzyme ranged between 1.5 to 1.75%(on oven dry germinated seeds) and it contained 12.65% nitrogen and 79.2% protein. Nitrogen was estimated by the Kjeldahl method (*23*) and protein was calculated by multiplying the nitrogen value by 6.25.

Enzymatic hydrolysis of the purified gums was carried out as described in the experimental part. The liberated monomer sugars were quantitatively estimated by the microchromatographic method (*22*). Efforts to relate scale readings of the Zeiss polarimeter (Hg-vapor lamp 566. 1 nm) with the amount of hydrolyzed galactose were unsuccessful. This system was too complex and gave unreliable results. The scale readings first rose and then dropped toward the negative side as the hydrolysis proceeded. The negative values were probably due to the mannan and enzyme (protein) contents in the reaction mixture.

LP_1 and GP_1 were used as substrates in the enzymatic hydrolysis. The amount of galactose liberated increased with the increase in reaction time (Fig. 1) and also with the enzyme concentration (Fig. 2) but decreased with the increase in the substrate concentration Fig 3). This decrease was probably due to the loss in mobility of the enzyme brought about by the high viscosity of the higher concentration of gum solutions. Maximum activity was found at pH 4.5 to 5.0 and 38°C (Fig. 4 and 5, respectively). The pH data and an extrapolation of the temperature curves showed that above pH 7.0 and 60°C the enzymes became inactive. It was found that upon heating. reaction mixture at 80°C for 5 min and then cooling down to 38°C the activity was not restored. Thus, permanent denaturation of the enzyme was brought about by heating. Reversibility of the enzyme activity due to pH was not tested. Optimum pH and temperature for hydrolysis were, therefore, fixed at 5.0 and 38°C, respectively. Other factors, such as reaction time, enzyme concentration, and substrate concentration are interdependent and so can be adjusted according to need.

Gel-electrophoresis showed that the isolated enzyme was a mixture of 8 proteins. However, the specificity of the enzyme mixture for alpha (1→6) linkages was surprisingly good as can be seen from

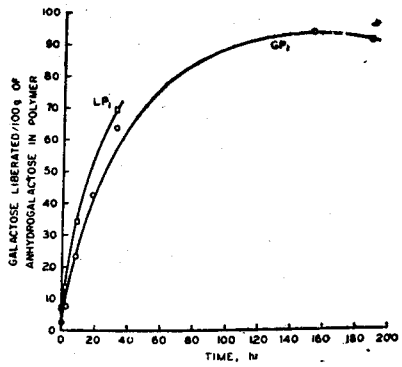
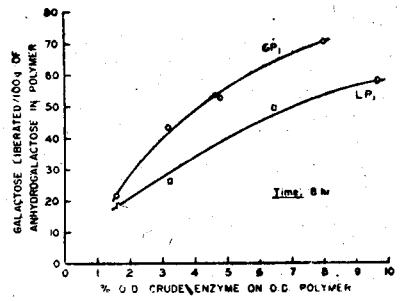


Fig. 1. Effect of time on the enzymatic hodrolysis of purified locust bean (LP_1) and guar (GP_1) gums.



Eig. 2. Effect of enzyme concentration on the enzymatic hydrolysis of purified locust bean (LP_1) and guar (GP_1) gums. Reaction time, 8 hr.

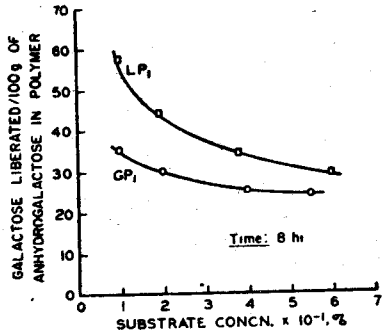


Fig. 3. Effect of substrate concentration on the enzymatic hydrolysis of purified locust bean (LP_1) and guar (GP_1) gums. Reaction time 8 hr.

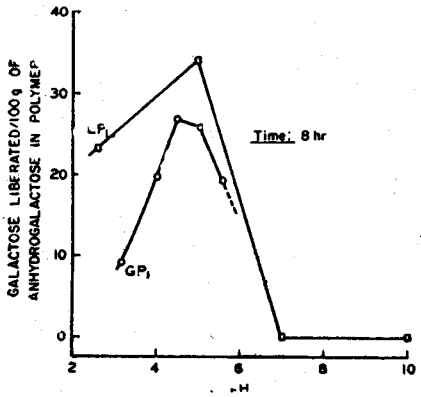


Fig. 4. Effect of pH on the enzymatic hydrolysis of purified locust bean (LP_1) and guar (GP_1) gums. Reaction time 8 hr.

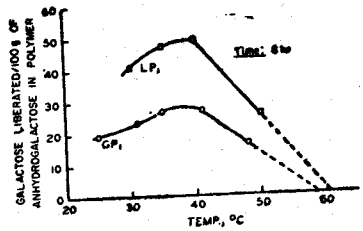


Fig. 5. Effect of temperature on the enzymatic hydrolysis of purified locust bean (LP_1) and guar (GP_1) gums. Reaction time 8 hr.

Table I. At low reaction time, only galactose is liberated. Free mannose is obtained if the hydrolysis is continued for 144 hours or more. In this study, LP_1 was more than 32 hours. It is expected that, at longer reaction time, mannose will also be liberated from LP_1. Maximum galactose and arabinose hydrolyzed from GP_1 were 95.2 and 98.2%, respectively. By extending the reaction time above 152 hours both of these values decreased. This decrease could be due to the transferring action of the enzyme (*12–15*). The nonhydrolyzable galactose, 4.8%, seems to be inaccessible to the enzyme and is, therefore, not affected. Arabinose is attacked only after more than 23% of the galactose has deen hydrolyzed. Glucose was not attacked.

TABLE I

Enzymatic Hydrolysis of Locust Bean (1P_1) and Guar (GP_1) Gnms

Gum	Gum concn., %	Enzyme[a] added, %	Reaction time, hr.	Galactose[b] cleaved, %	Mannose[b] cleaved, %	Arabinose[b] cleaved, %
Locust bean	0.485	1.65	35 (min)	7.4	—	—
	0.485	1.65	2	13.7	—	—
	0.485	1.65	8	34.1	—	—
	0.485	1.65	32	69.6	—	—
Guar	0.505	1.58	30 (min)	2.9	—	—
	0.505	1.58	2	7.5	—	—
	0.505	1.58	8	23.1	—	—
	0.505	1.58	18	42.8	—	71.0
	0.505	1.58	32	64.0	—	83.6
	0.505	1.58	152	95.2	2.6	98.2
	0.505	1.58	188	93.4	5.4	94.8

[a]On the basis of oven-dried gum.
[b]On the basis of total anhydrosugars (galactose, mannose, or arabinose), present in the gum, respectively,

During the hydrolysis of galactose from galactomannan a white precipitate was obtained indicating the aggregation of the linear mannopyranosyl chains into larger water-insoluble structures. The mannan precipitate was thoroughly washed with distilled water and freeze-dried. Upon total hydrolysis (*22*) the precipitate yielded 4.85 g galactose, 96.1 g mannose (mannose-to- galactose ratio 19.8), and 1.74 g glucose per 100 g dry basis of the precipitate.

X-ray diffractograms for LP_1, GP_1, LP_1H (hydrolyzed locust bean gum), GP_1H (hydrolyzed guar gum) and ivory nut mannan were prepared. LP_1 and GP_1 gave typical amorphous-type curves (Fig.6) whereas LP_1H and GP_1H curves showed peaks at the same 2θ value as that of ivory nut mannan but were broader indicating a greater imperfection of the crystal phase as compared to the ivory nut mannan. The peak between 2θ values of 26 and 27° is missing in the case of LP_1H and is present in the other two mannans. This difference in x-ray pattern is probably due to the difference in the constituents of each mannan because LP_1H upon hydrolysis gave only galactose and mannose whereas GP_1H gave galactose, mannose and glucose and ivory nut mannan gave galactose ,mannose.glucose, rhamnose, and xylose. The change of LP_1 and GP_1 from amorphous to the crystalline state and their similarity with the naturally occuring ivory nut mannan indicates the specificity of the enzyme for alpha (1→6) linkages. The crystallinity

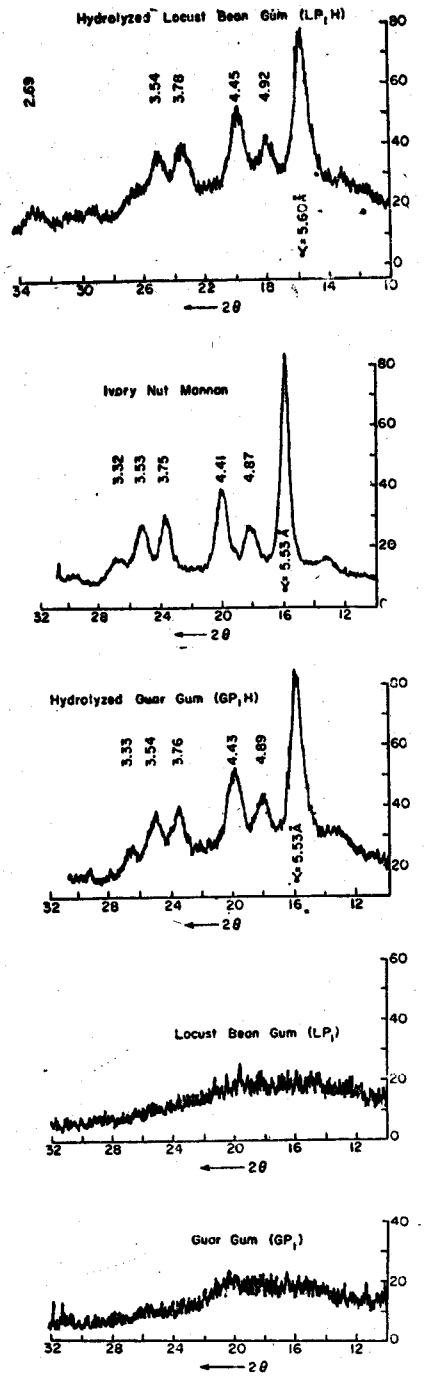


Fig. 6. X-ray diffractograms of original and modified gums. (LP_1H, GP_1H and mannan at scale factor of of 8. LP_1 and GP_1 at scale factor of 2. Orindate scale shows recorded intensities.)

is developed due to the attractive Van der Waals forces which become more effective as the polymer becomes more linear and fewer blocking galactose groups are present.

Molecular weight of the nitrated GP_1 and Gp_1H (GP_1 Hydrolyzed for 188 hr) products were determined by a high-speed membrane osmometer using ethyl acetate as the solvent. The number average molecular weight of the nitrated GP_1 was found to be 1.86×10^6 (original GP_1 $1.01 \times 10^6 = 6230$DP) and of nitrated GP_1H 17,000 (original GP_1H 9260=57.2 DP).

Although the 188 hr GP_1H was somewhat degraded, the nitration of this material was carried out because the GP_1H (hydrolyzed for 144 hr) was used up for acetylation purposes and the acetylated material was found to be insoluble in acetophenone, acetonitrile, 1-1-2 trichloroethane, dimethylsormamide and dimethylsulfoxide.

It is expected that if the hynrolysis time c n be kept below 144 hr, a less degraded mannan precipitate can be obtained-

Experimental

Isolation Of Alpha-Galactosidase

Isolation of the enzyme was carried out from different sources such as molds (grown on different seeds) and sprouted guar seeds.

From Molds—Seeds like Huizache (*Cesalpinia cacaloca*), Tara (*cesalpinia spinosa*), Flame tree (*Dclonis regia*), Palo Varde (*Cercidium torreyanum)* Guar (*Cyamopsis tetragonoloba)*, and Locust bean (*Ceratonic siliqua* L.),were kept

under moist conditions at 38°C. Molds which appeared on the seeds after 30 hours were fully developed after 5 days. Purification of the molds was carried out by the dilution–shake method *(24)* and the pure molds were cultured on a medium containing 19.5 g Bacto[1], 1.0 g Agar and 500 ml distilled water. Cultured molds were further purified and then allowed to grow on a medium containing 4.6 g galactomannan, 1.0 g sodium nitrate, 1.0 g glucose. 0.25 g magnesium sulfate, 0.5 g potassium sulfate, 0.25 g potassium chloride, 2.0 g Agar and 500 g distilled water. Molds were repeatedly purified and acclimated on the above media containing the galactomannan gum by following the above procedure Pure molds were incubated for seven days, ground in a pestle and mortar with increasing amounts of distilled water and centrifuged. The clear extract was tested for the enzyme activity in the Ostwald Viscometer size 300. Five ml of the extract were added to the viscometer containing 10 ml of 0.1% locust bean gum solution and kept at 30°C in a water bath. Efflux time was noted every half hour.

From Sprouted Guar Seeds—

During the process of germination the galactomannan polymer in guar seeds is enzymatically hydrolyzed and consumed. The consumption of this reserve food continues until the germinating seed starts producing its own food. Therefore, the percentage of the respective enzyme should show a maximum during the course of germination. Experiments showed that maximum enzymatic activity was present when the sprouts were between 3-4 cm long.

Guar seeds were soaked in tap water for 2 days, washed well and allowed to germinate at 30°C between moist bleached pulp sheets (sheet covering the seeds had punched holes).At the completion of the germination period seeds having sprout length of 3-4 cm were hand picked and alternately frozen and thawed at least twice (maximum thrice). to break up the tissue structure. Two to three grams of the thawed seeds were used for moisture determination and the rest were weighed, processed at high consistency with toluene-water in a Warning Blendor for 1 to 2 min and stirred slowly at room temperature for 6–7 hr. The slurry was centrifuged in an ultracentrifuge Model L–2 at 15,000 rpm for 15 to 20 min. The cleared light-colored extract was filtered through a glass fibre filter and kept for precipitation purposes.

Precipitation And Purification Of Alpha-Galactosidase.

Belfrich and Vorsatz *(10)* had used 2% tannin solution for the precipitation of enzyme but enzyme obtained using their method was not soluble in water. It, however had the desired activity. Whistler, *et al. (25)* precipitated the enzyme with ammonium sulfate and then dialyzed to remove excess sulfate ions. We prepared the enzyme by both the above methods. Enzymes prepared by the former method hydrolyzed only galactose and by the latter method both galactose and mannose from the locust bean galactomannan. Therefore, the following two-step precipitation process was developed using tannic acid for precipitation.

Thirty ml of 2% tannic acid was added to 500 ml of the clear but crude enzyme extract and the light brown precipitate thus obtained was centrifuged at 13,000 rpm in the beta-centrifuge for 15 min. (Filtration at this stage is not advisable as the pecipitate sticks to the filter paper and is very difficult to filter). This precipitate showed no enzyme activity. The supernatant clear liquid was filtered through a glass fiber filter and the precipitation was completed with 2% tannic acid solution. The precipitate was centrifuged at 13,000 rpm in the beta-centrifuge for 15 min, washed with acetone, decanted,kneaded with acetone in a mortar and pestle and kept overnight under acetone. It was then filtered on an ordinary filter paper, washed with acetone until free of tannic acid (until the filtrate did not give color with ferric chloride) and vacuum dried, The dried precipitate was suspended in distilled water for 20 min. and the suspension was centrifuged at 10,000 rpm for 10 min. The supernatant liquid was filtered through a glass fiber filter and the clear filtrate was freeze–dried. The dried powder was easily soluble in water and had alphagalactosidase activity. Yield on the basis of oven-dried seeds was 1.5 to 1.75%.

[1]Bacto potato dextrose agar dehydrated. Detroit, Mich., Difco Labs Ind.

Polyacrylamide-Gel Disk Electrophoresis

Gel-electrophoresis of the enzyme was carried out by the method described by Davis *(26)*. Two mg of enzyme were dissolved in 0.2 ml of large pore solution LPS (*A*); 0.15 ml of *A* was diluted with 0.15 ml LPS (*B*); 0.15 ml of *B* was diluted with 0.15 ml of LPS (*C*); similarly *C* was diluted to *D*. Only 0.1 ml each of *A*, *B*, *C*, and *D* was used per tube for gel-electrophoresis.

Enzymatic Hydrolysis Of The Galactomannan Gums

All hydrolysis experiments were carried out under sterile conditions. Galactomannans of locust bean (LP_1) and guar (GP_1) seeds were used as the substrate.

Twenty-five to fifty milliliters of 0.125 to 0.75% gum solutions were placed in 250-ml Erlenmeyer flasks maintained at 35°C in a water bath. Ten ml of acetic acid-sodium acetate buffer of pH 5.0 and 4 ml of 1.6 mg/ml enzyme solution were added in succession to each flask. After the reaction had reached the desired stage, the enzyme was denatured by heating the flask at 80°C for 5 min. Liberated monomer sugars were quantitatively estimated by the microchromatographic method *(23)*. The insoluble mannans, obtained as a white pricipitate during enzymatic hydrolysis, were thoroughly washed with distilled water and freeze-dried for further analysis.

Effects of variables like time (range 30 min to 188 hr), temperature (25 to 50°C), pH (3 to 10), enzyme concentration (1.5 to 10%) and substrate concentration (0.1 to 0.6%) on LP_1 and GP_1 were studied following the above method of hydrolysis and by changing the desired variable but keeping the others constant.

Crystallinity By X-Ray Diffraction

X-ray powder diagrams were prepared for GP_1, LP_1, GP_1H, LP_1H, and ivory nut mannan[2], using a Norelco x-ray diffractometer and a Cu-tube with Ni-filter for CuKα radiation ($\lambda = 1.5418$ A) at 35 kv and 20 ma. The scanning rate from 10 to 30° 2θ was 0.5° per minute.

Molecular Weight Determination

Acetylation and nitration of GP_1 and GP_1H were carried out by the methods suggested by Timell *(27)* and Golbfrank *(28)*, respectively. Degree of acetylation was tested by IR-spectroscopy. Molecular weights were determined by the high-speed membrane osmometer, Model 501 (Mechrolab Inc.) and the number average molecular weight was calculated by the formula.

$$M_n = \frac{RT}{\pi/C}$$

where π=osmotic pressure

C=concentration

R=gas constant

T=absolute temperature

M_n=number average molecular weight.

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