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A β-1, 6-Glucan 6-Glucanohydrolase from *Gibberella fujikuroi**

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Abstract

The general properties and action pattern of a β-1, 6-glucan hydrolase of *Gibberella fujikuroi* were studied. The β-1, 6-glucan hydrolase production is inducible in *G. fujikuroi* culture with lutean or gyrophoran as good inducers. The enzyme is partially induced with glucose. A β-1, 6-glucan hydrolase has been purified from the culture filtrate of the fungus, using ion-exchange and gel-filtration chromatographys, which is free from both β-1, 3-glucan and β-glucoside hydrolases. The purified enzyme is homogeneous on analyses by ultracentrifugation and by disc-electrophoresis. The optimal pH of the purified enzyme for the hydrolysis of lutean is 5.0. The activation energy for the inactivation of the enzyme is proved to be lowest at the stable pH, 7. The purified enzyme is a β-1, 6-glucan 6-glucanohydrolase, hydrolyzing lutean, gyrophoran, and gentio-oligosaccharides with the degree of polymerization greater than four. The final products from lutean consist of gentiotriose, gentiobiose, and glucose in the ratio of about 3 : 19 : 3 as glucose. The apparent K_m values of the enzyme for lutean and gentiotetraose are 1.2×10^{-7} M, and 1.4×10^{-5} M, reaspectively.

Introduction

A considerably wide distribution of β-1, 6-glucosidic linkages, and the enzyme which hydrolyzes it in nature, have been recognized recently.[1-3] However, information on the enzyme which hydrolyzes β-1, 6-glucan is not sufficient to enable the discussion of the mechanism of the enzymic attack on the polysaccharides or to use it for a study of their structure.

Gibberella fuijkuroi has been found to be one of the best producers of β-1, 6-glucanase.[1] The culture filtrate of the fungus contains β-glucosidase components and occasionally a small amount of β-1, 3-glucanase, also. The activities of both were found to be easily removable by ion exchange chromatography. The present report describes the purification and characteristics of the β-1, 6-glucan hydrolase of the fungus.

Materials and Methods

1. Materials *Gibberella fujikuroi*, G—3** has been kindly supplied by the Institute of Agricultural Technology, Nishigahara, Tokyo. Lutean was prepared from the culture

* Enzymic Hydrolysis of Glucans Containing β-1, 3- and β-1, 6-Linkages (II)

** Science bureau strain number 1082

filtrate of *Penicillium aculeatum* var. *apiculatum*.[1] 'Gyrophoran', another β-1,6-glucan*, was extracted from *Gyrophora esculenta*, saponified, and purified. Pachyman was prepared from bukuryo.[1]** β-1,6-Oligoglucosides were prepared by acid (1 N H_2SO_4, 1 hr. in a boiling water bath) or enzymic hydrolysis of the glucans. Each oligoglucoside was prepared by carbon column chromatography with an increasing concentration of aqueous ethanol as a solvent.

2. Methods Cultivations were carried out at 30° on a reciplocal shaker in a shaking flask (80 ml/300 ml) or in a test tube (10 ml/30 ml). The composition of the basal medium for cultivation of the fungus was the same as described in a previous paper.[1] β-Glucan was used as both the inducer and the carbon source. The pH of the medium was adjusted to 5. Mycerial growth was determined by the protein concentration of a supernatant solution of a broken cell suspension disintegrated for 5 min in a Universal homogenizer (Nihonseiki, Type H-1). Protein concentration was determined by the method of Lowry or by absorbance at 280 nm. Ultracentrifugal analysis was made in a double sector cell by the synthetic boundary method at a speed of 55,430 rpm (226,712.5×g after 25 min at the peak of the enzyme) by the Hitachi UCA-1 with uv absorption equipment. Reducing sugar determination, enzyme assay, and ordinary paper chromatographic analysis were carried out as described previously.[1] An additional paper chromatographic technique similar to the 'oligosaccharide map' method[5] was employed for the study of the action pattern of the purified enzyme. A gentio-oligosaccharide mixture (partially acid-hydrolyzed lutean) was applied near a corner of a large sheet (60×60 cm) of Toyo No 51 filter paper (indicated as the original point in Fig 12.), and separated by the descending technique. After the first irrigation, the area of the dried paper containing the resolved components was sprayed with purified enzyme of an appropriate concentration in a dilute buffer solution of pH 5, and the damp paper was incubated (15 to 60 min) in a humid chamber to facilitate the enzyme reaction. Subsequently to drying, the paper was irrigated in the second direction. Reference sugars were spotted each time for the first and second developments. A guide strip for the first run was cut off before the second run. Sugar detection on the paper was performed in the same way as in ordinary paper chromatograms. Disc-electrophoresis was carried out according to the standard method (7% acrylamide, Tris-HCl buffer, pH 9.5). The protein band in the gel was located by Coomasie Brilliant Blue R 250 staining. Electrical resistance or conductivity of the column eluate was determined by a Toa conductivity meter, type PM-3M.

Results

1. Production of β-1,6-glucan-hydrolase Hydrolase activities for β-1,6-glucan and related substrates of cultures grown on several different carbon sources were examined after 60, 140, and 190 hours' cultivation (Table 1.). Protein concentration in the homogenized cultures indicates that these carbon sources are all good for mycelial growth. β-1,6-Glucanase activity appeared best when the fungus was grown on gyrophoran. A comparable activity, but low in specific activity, appeared when the fungus was grown on glucose. However, the enzymic activity did not appear at any stages of the growth on

*) Tentative name for a β-glucan extracted form *Gyrophora esculenta*.[1] Shibata, *et al.*: Chem. Pharm. Bull., 16, 1639 (1968).

**) Dried sclerotium of *Poria coccos* Wolf, an ingredient of Japanese folk medicine, contains pachyman about 94% of its weight. Purchased from Kinokuniya-herb pharmacy, Kanda, Tokyo, Japan.

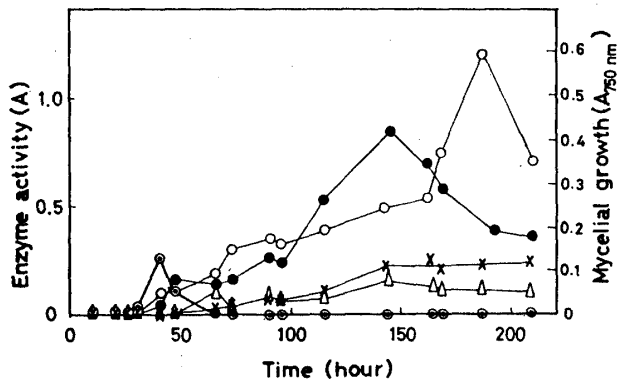
Table 1. Effect of the carbon source on the production of hydrolases for β-1,6-glucan, β-1,3-glucan, and β-glucoside in a *Gibberella* culture.

	Mycelial growth (Protein concn[c])			Enzyme activity (units/ml)[b]								
				β-1,6-Glucanase			β-1,3-Glucanase			β-Glucosidase		
Incubation time (hr) / Carbon source[a]	60	140	190	60	140	190	60	140	190	60	140	190
Succinate	0.28	0.16	0.14	0.01	0.00	0.01	0.04	0.02	0.01	0.18	0.22	0.24
MSG	0.48	0.32	0.28	0.04	0.02	0.00	0.04	0.04	0.01	0.25	0.23	0.22
Glycerol	0.38	0.37	0.34	0.00	0.00	0.01	0.02	0.03	0.03	0.04	0.18	0.23
Glucose	0.50	0.55	0.38	0.01	0.37	0.33	0.03	0.07	0.17	0.18	0.28	0.29
Gyrophoran	0.25	0.24	0.19	0.13	0.39	0.57	0.02	0.05	0.03	0.20	0.21	0.25

a: The concentration of the main carbon source is 0.2%.
b: The change in absorbance during one hr was used as a measure of enzyme activity. 43.2 units of glucanase and 72.6 units of glucosidase activities correspond to the unity proposed by IUB (micromole product/min).
c: Absorbance at 280 nm determined by a Hitachi 124 photoelectric spectrophotometer in a cell with a 1.0 cm light path length.

monosodium glutamate (MSG), succinate, or glycerol. β-1,3-Glucan and β-glucoside hydrolases production was not so much affected by the choice of carbon source and seems to be almost simultaneous with mycelial growth.

Mycelial growth (total protein) and enzyme production as a function of time are given in Fig. 1 together with the reducing sugar formation in the culture filtrate of the fungus grown on gyrophoran. A maximum of protein concentration was observed after 144 hr. β-1,6-Glucanase activity of the culture reached a maximum 30—40 hr after that of the protein concentration. An increase and then an immediate decrease in the reducing sugars in the culture were observed in an early stage of the cultivation.



●—● Mycelial growth (Lowry $A_{750\ nm}$).
⊙—⊙ Total reducing sugars in culture filtrate (Nelson $A_{500\ nm}$).
○—○ β-1,6-Glucan hydrolase
△—△ β-1,3-Glucan hydrolase
×—× β-Glucoside hydrolase

Fig. 1. Cultivation time and enzyme production. Cultivation was carried out at 30°C on a reciprocal shaker in a shaking flask (500 ml) containing 100 ml of culture fluid with 2% gyrophoran as the main carbon source. An aliquot was taken out at the indicated time, disintegrated, and assayed for protein, enzymes, and reducing sugars. The enzyme solution was added to an equal volume of substrate solution (0.3% lutean or pachyman, or 0.002 M *p*-nitrophenyl β-glucoside).

2. Enzyme purification Crude enzyme was prepared from a culture filtrate of *G. fuji-*

kuroi grown on a medium containing lutean or gyrophoran as the main carbon source. Evaporation under reduced pressure below 30°C and dialysis against dilute neutral phosphate buffer in the cold were repeated alternately. The resulting enzyme concentrate was stored frozen or lyophilized. Fractional precipitation of the culture-filtrate concentrate by ammonium sulfate salting out was attempted. The related enzyme activities, however, behaved similarly to β-1,6-glucan hydrolase, as shown in Fig. 2. Purification by ion-exchange chromatography was performed as follows: The crude enzyme concentrate was placed on a DEAE-cellulose column equilibrated with 0.005 M potassium phosphate buffer, pH 8.0, washed with the same buffer, and eluted by the buffer with a gradually increasing concentration up to 0.1 M. A small amount of β-1, 3-glucanase eluted the first, then β-1,6-glucanase. β-Glucosidase remained on the column until the application of a higher ionic strength of buffer (Fig. 3). A trace of β-1, 3-glucanase which contaminated the β-1, 6-glucanase fraction was removed by CM-cellulose column chromatography. A CM-

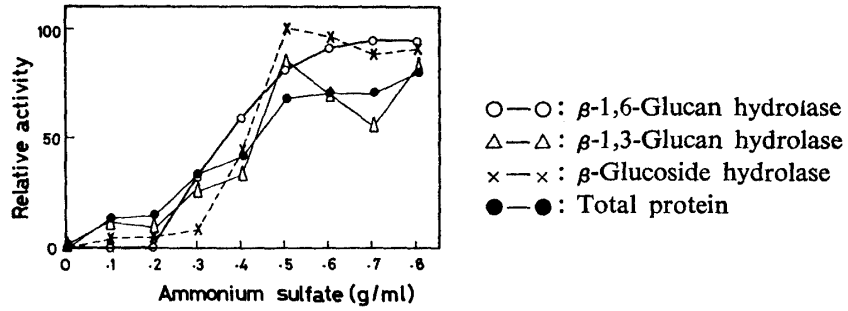


Fig. 2. Ammonium sulfate concentration and the enzymic activity of the precipitate salted out. An appropriate amount of powdered ammonium sulfate was dissolved into each 10 ml portion of dialyzed enzyme concentrate, and these were allowed to stand overnight at room temperature. The precipitate was collected by centrifugation, washed with aqueous ammonium sulfate of the same concentration, dissolved in saline solution, dialyzed, and assayed. The relative activity to that of the original enzyme solution is indicated.

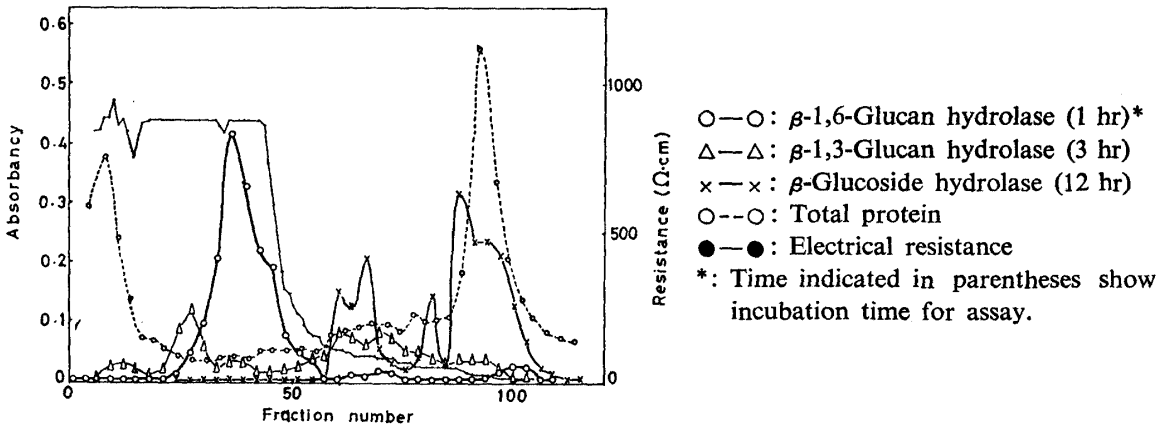


Fig. 3. Separation of enzyme components of *G. fujikuroi* culture filtrate on a DEAE-cellulose column. A sample was put on a column (2.5×40 cm) equilibrated with 0.005 M potassium phosphate buffer, pH 8.0, washed with the same buffer, eluted with buffer of a gradient concentration from 0.005 to 0.1 M, then with a KCl-gradient in the buffer to 1 M.

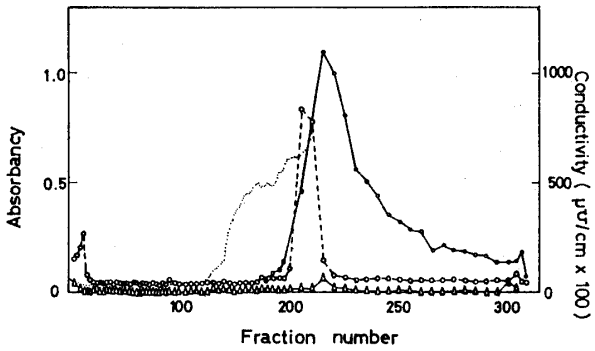


Fig. 4. CM-cellulose column chromatography of β-1,6-glucan hydrolase of *G. fujikuroi*. A concentrated and dialyzed preparation from a DEAE-cellulose column (fractions no. 33-75 of Fig. 3) was put on a column (2.5×40 cm) equilibrated with 0.005 M potassium acetate buffer, pH 5.0, washed with the same buffer, eluted with a gradient concentration of the buffer to 0.1 M, then with a KCl-gradient to 1 M.

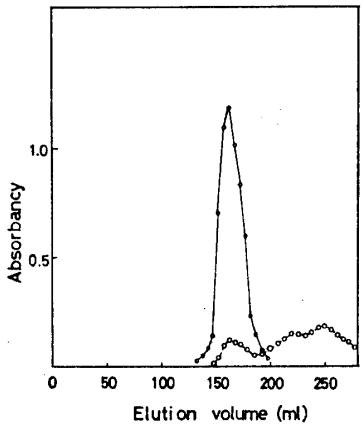
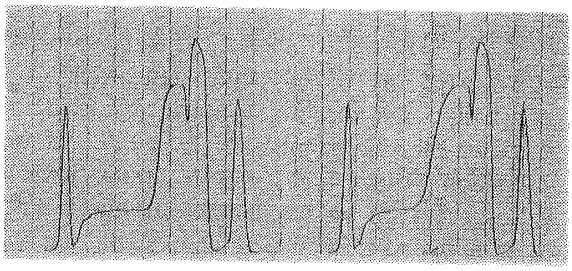


Fig. 5. Biogel P-100 column chromatography of *Gibberella* β-1,6-glucanohydrolase. A concentrated and dialyzed (0.85% saline) fraction from an ion-exchange column (Fr. no. 220-300 from a CM-cellulose column) was put on a Biogel column (2×150 cm) and developed with the saline solution.
○—○: β-1,6-Glucan hydrolase
○···○: Total protein

Table 2. Purification profile of a β-1,6-glucan glucanohydrolase from the culture filtrate of *G. fujikuroi*.

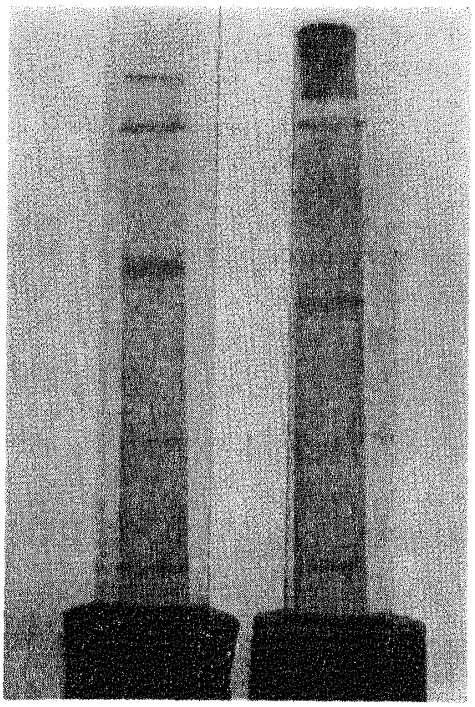
Preparation	Enzyme activity		Protein		Specific activity[b]
	Units 0.1 ml	Total units	A_{280} nm	Total protein[a]	
Culture filtrate (1,000 ml)	1.800	1.8×10^4	2.00	2.0×10^3	0.900
DEAE-cellulose chromatography fraction no.					
33—75		1.44×10^4		467	3.08
46	0.845	33.80	0.085	0.340	9.94
CM-cellulose chromatography fraction no.					
220—300		8.51×10^3		92.8	9.17
221	0.333		0.010	0.040	33.3
Biogel P-100 chromatography elution vol.					
148—190 (ml)		8.40×10^8		24.5	34.3[c]

a: $A_{280\,nm} \times$ volume
b: Total activity/Total protein
c: The purified enzyme produces reducing sugars corresponding to 4.43 μ moles of glucose/min/mg protein (supposing that $A_{280\,nm}$ of the enzyme equivalent to that of BSA).



a b

Fig. 6. Ultracentrifugation of purified *Gibberella* β-1,6-glucan hydrolase. Speed, 55,430 rpm (226,712.5×*g* after 25 min at the peak of the enzyme); protein concentration, about 1%; temperature, 25°C; solvent, 0.1 M potassium phosphate buffer, pH 7.
a: 0 time b: 25 min



(b) (a)

Fig. 7. Disc-electrophoresis of crude (a) and purified (b) *Gibberella* β-1,6-glucanohydrolase preparations. Gel, 7% acrylamide gel; buffer, Tris-HCl, pH 9.5; current, 24 mA; voltage, 150 volts; time, 4 hr; staining, Coomasie Brilliant Blue R-250.

cellulose column equilibrated with 0.005 M sodium acetate buffer, pH 5, was loaded with the enzyme fraction, and eluted with the same buffer in an increasing concentration from 0.005 to 0.1 M (Fig. 4.) The enzyme was further purified by Biogel P-100 chromatography (Fig. 5). The purification of the enzyme is summarized in Table 2. The molecular weight estimated from the elution volume of the enzyme is about 3.7×10^4. The purified β-1,6-glucanase preparation hydrolyzed neither pachyman nor *p*-nitrophenyl β-glucoside even after long (72 hr) incubation at 40°C. The homogeneity of the preparation was established by ultracentrifugal and disc-electrophoretic analyses (Figs. 6 and 7).

3. General properties of the enzyme The effect of the pH of the reaction mixture on the lutean hydrolyzing activity of the enzyme at 40°C was examined. The highest activity was observed at pH 5.0 as shown in Fig. 8.

The stability of the enzyme in solutions of different pH was investigated at 30, 40, 50, and 60°C (Fig. 9). Solutions of the enzyme were titrated to the desired pH by the careful addition of 1 M acetic acid or NaOH, and incubated. After a predetermined period, aliquots were removed and diluted with a pH 5.0 "stopping" buffer (0.1 M potassium acetate), and assayed after 10 min. Residual activity after 30 min incubation is

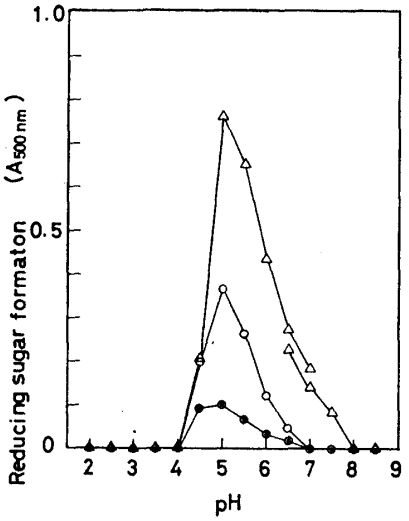


Fig. 8. Effect of pH on β-1,6-glucanohydrolase activity. The reaction mixtures consisted of 0.5 ml 2% gyrophoran, 1.0 ml 0.1 M sodium acetate (pH 3—7) or citrate (pH 2—3, 7—8.5) buffers and 0.5 ml enzyme solution. After incubation at 40°C, 0.5 ml of each reaction mixture was removed, neutralized and assayed for total reducing sugars.
⊙—⊙ 30 min
○—○ 1 hr
△—△ 21 hr

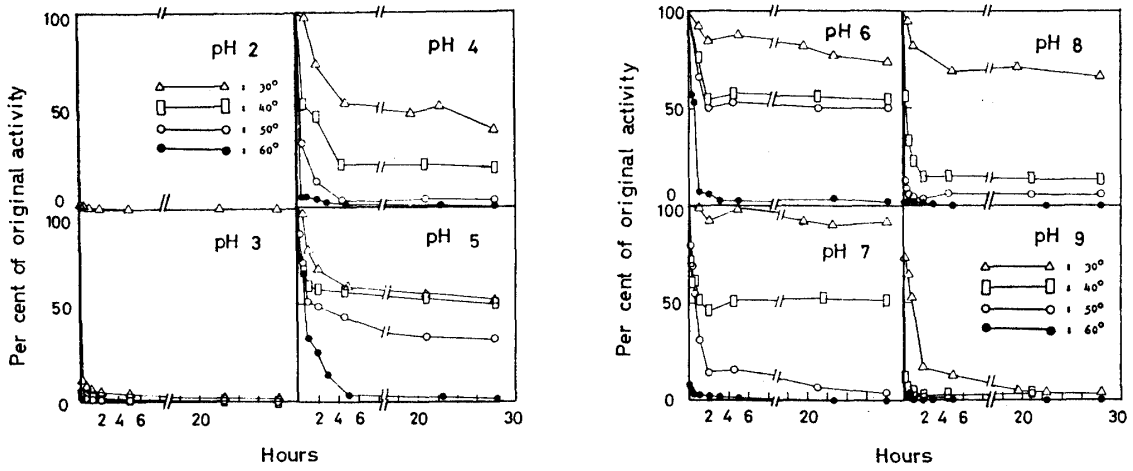


Fig. 9. Effect of pH on enzyme stability. After each incubation, the enzyme solution was adjusted to pH 5 with acetic acid or NaOH, and assayed. See text for procedure.

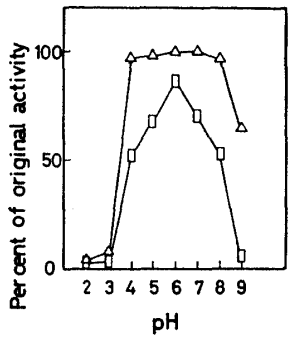


Fig. 10. Effect of pH on the enzyme stability; residual activity after 30 min incubation. Temperature of exposure to the pH indicated: △—△, 30°C; □—□, 40°C.

summarized in Fig. 10. *Gibberella* β-1,6-glucan hydrolase was stable for a long time at 30°C at pH 6, 7, and 8. At 40°C, the activity decreased rapidly, however; about 50 % of the original activity remained for more than 30 hr at pH 5, 6, or 7. At 50°C, about

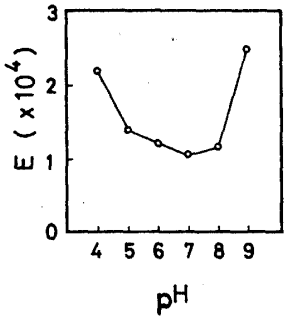


Fig. 11. Apparent activation energy for inactivation of *Gibberella* β-1,6-glucan hydrolase at different pH values. Activation energy (E) was calculated from k 40°/k 50° for initial inactivation velocities.

40 to 50% of the activity survived for more than 30 hr at pH 5 and 6. At 60°C the enzyme activity was lost very rapidly at any pH, but rather slowly at pH 5.

The apparent activation energy (E) for inactivation of the enzyme, calculated from the initial rate, changed with pH (Fig. 11), and was lowest at pH 7.

4. Hydrolysis of substrates Lutean, gyrophoran, and gentiooligosaccharides were subjected to the action of the purified enzyme and the hydrolysis products were analyzed. Lutean was incubated with the enzyme in 0.05 M sodium acetate buffer, pH 5, at 40°C. After an appropriate time of incubation, aliquots were taken and analyzed by the Somogyi-Nelson method for reducing sugars and by paper chromatography for the component sugars. Higher oligosaccharides with a degree of polymerization greater than four were detected on a paper chromatogram of the hydrolyzate at earlier time of incubation with the enzyme. The final products, after an excess time of incubation (4 days under toluene), were analyzed by Biogel P-2 column chromatography (1×170cm) (Table 3). About 90

Table 3. Biogel P-2 column chromatography of the final hydrolysis products from lutean. About 5 mg of lutean and 50 units of enzyme were used.

	Amount recovered (μg)	Ratio as glucose (%)
Polymer	491	10
Oligomer and monomer	4,445	90
Gentiotriose*	476	12.1
Gentiobiose*	2,986	11.7
Glucose*	457	11.7

*: Data from the rechromatography of the oligomer and monomer fraction, started with about 4 mg.

% of the product sugars consisted of glucose, gentiobiose, and gentiotriose in the ratio of 3 : 19 : 3 as glucose. The rest, 10 % of the hydrolyzate, the faster moving component in the Biogel column, have not been characterized. Gyrophoran was similarly hydrolyzed by the enzyme.

The action pattern of the enzyme on each gentio-oligosaccharide was examined using two dimensional paper chromatography interspersed with an enzymic reaction on the paper. The results indicated that the enzyme hydrolyzes gentio-oligosaccharides of d.p. above four, and gentiobiose and gentiotriose are not hydrolyzed (Fig. 12). Only gentio-oligosaccharide was detected on paper chromatograms of the hydrolysis products of lutean by the purified β-1,6-glucanohydrolase. However, the possibility that the enzyme might

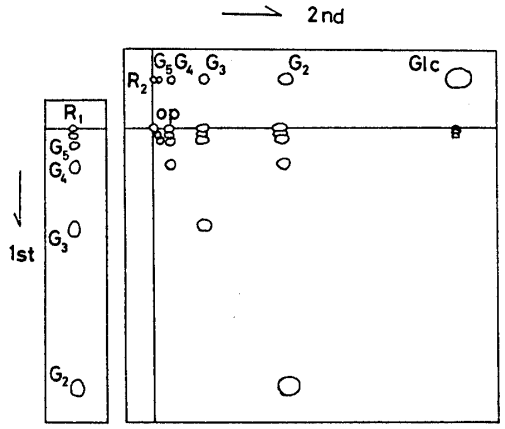


Fig. 12. Action pattern of *Gibberella* β-1,6-glucanase on gentooligosacchariides. A gentiooligosaccharide mixture was applied at OP as the enzyme substrate, and as reference sugars, R_1, for the first irrigation indicated by an arrow 'lst'; and R_2, for the second irrigation '2nd'. Developed in butanol/pyridin/water/benzene (5:3:3:1). Detected by silver nitrate method. Glc, glucose; G_2, gentiobiose; G_3, gentiotriose; G_4, gentiotetraose; G_5; gentiopentaose.

transfer a glycosyl group[6] was checked, employing 1% ^{14}C-labeled glucose as an acceptor, and lutean and several gentio-oligosaccharides as donors. No radioactive oligosaccharide was detected on radioautograms of the enzymic reaction mixture tested.

The effect of substrate concentration on the enzyme activity was studied determining the rate of hydrolysis of lutean and gentiotetraose at a series of concentrations. Apparent K_m values were determined by a double reciprocal plot of the observed values to be 1.2×10^{-7} M for lutean, the molecular weight of which was assumed as 6.5×10^{5}[7] and 1.4×10^{-5} M for gentiotetraose.

Discussion

β-1,6-Glucanase production in *G. fujikuroi* is dependent on the carbon source and is typically inducible. Lutean and Gyrophoran have a high inducing activity. Glucose also has a certain inductive effect; its repressive effect on the enzyme production is not complete, as has already been shown in a previous paper.

Several microorganisms have been found which produce a high activity of β-1,6-glucanase in a previous screening,[1] however, most of them produce β-1,3-glucanase also. *G. fujikuroi* seemed to be one of the rare fungi which produce a high level of β-1,6-glucanase and a negligible level of β-1,3-glucanase. However, in the subsequent experiments the fungus has been found to occasionally produce a noticeable amount of β-1,3-glucanase. A time course study on the growth and the enzyme production of *Gibberella* made this clear. β-1,3-Glucanase production occurs simultaneously with mycelial growth and decreases with ease, and seems to be constitutive; β-1,6-glucanase production, on the contrary, follows about 30 hr later, and by that time β-1,3-glucanase activity drops sometimes to a negligible level.

Oscillation phenomena were observed in the production of these enzymes and even mycelia of (Fig. 1). It is interesting that recent theory[8] on the formation of inducible enzyme systems or microbial growth describes various types of oscillating processes under certain conditions. The rapid increase and decrease of the reducing sugars in the early stage of the culture seem to indicate that the release of free sugars by the constitutive or primarily induced enzyme overcomes temporarily their consumption by the fungus. However, once mycelial growth starts, the released sugars are taken up by the fungus immediately.

The β-1,6-glucan hydrolase from the *G. fujikuroi* culture filtrate has been purified in

a homogeneous state. The specific actvity of the purified enzyme, however, compared to that of the starting culture filtrate, was not so high. The synergistic action of β-1,6-glucan hydrolase and β-glucoside hydrolase contained in the starting preparation makes the activity higher than that of the former alone. This seems to be the reason for such a small increment in the specific activity of the enzyme during the purification. Under the conditions of the cultivation, *Gibberella* seems to produce a relatively small amount of protein outside the cells; this may be another reason. That only a single protein band was detected with Coomasie Brilliant Blue on disc-electrophoresis provides good support for the homogeneity of the purified enzyme because of the high sensitivity of the dye for proteins.

In the stability experiments, the residual enzyme activity often leveled off at a semistable state of about 50% of the original, similar to the cases of the inhibition of yeast sucrase.[9] Two stages of stable states in the inactivation processes can be expected. However, a possible microheterogeneity of the enzyme as a mixture of stable and labile components cannot be eliminated in spite of the homogeneity proofs.

The intermediary production of the higher oligosaccharides during the hydrolysis of β-1,6-glucans indicates the enzyme to be a glucanohydrolase. This was ascertained by the negative data for transglycosylation by the enzyme, which might produce such oligosaccharides from the lower ones.[6]

Glucanohydrolases can be classified into several groups based on their activity towards oligosaccharides. The glucanohydrolases which have a high affinity for the oligosaccharides seem to be more saccharogenic, less random, and the ones with a lower affinity seem less saccharogenic, more random, as in the cases of cellulase[10] and α-amylases. *Gibberella* β-1,6-glucanohydrolase has no activity toward gentiobiose and gentiotriose, and hydrolyzes gentiotetraose slowly.

The main product of lutean hydrolysis is gentiobiose with glucose and gentiotriose in small and almost equal amounts as glucose. A minor component of the final product is the polymer fraction from Biogel chromatography. Although the higher polymers have not been characterized, it seems to be the residual limit polymers which have some resistant structure for β-1,6-glucanohydrolase.

Lutean and gyrophoran, both consisting mainly of β-1,6-linked glucose and having some differences in their molecular weights, hydrolyzed in almost the same fashion and in the same final degree of hydrolysis (about 40% as glucose).

The effect of the higher substrate concentration on the enzymic activity is inhibitory so that the velocity vs. concentration curves make a bell shape, and *Km* represents an apparent value.

Specificity of the *Gibberella* β-1,6-glucanohydrolase for the other glucans containing both β-1,6-and β-1,3-linkages will be described in subsequent papers.

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