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PURIFICATION AND SOME ENZYMATIC PROPERTIES OF THE CHITOSANASE FROM *BACILLUS* R-4 WHICH LYSES *RHIZOPUS* CELL WALLS

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Summary

A strain of *Bacillus* sp (*Bacillus* R-4) produces a protease and a carbohydrolase both of which have the ability to lyse *Rhizopus* cell walls. Of the enzymes, the carbohydrolase has been purified to an ultracentrifugally and electrophoretically homogeneous state, and identified as a chitosanase. The enzyme was active on glycol chitosan as well as chitosan. Molecular weight of the purified enzyme was estimated as 31 000 and isoelectric point as pH 8.30. The enzyme was most active at pH 5.6 and at 40°C with either *Rhizopus* cell wall or glycol chitosan as substrate, and was stable over a range of pH 4.5 to 7.5 at 40°C for 3 h. The activity was lost by sulfhydryl reagents and restored by either reduced glutathione or L-cysteine. An abrupt decrease in viscosity of the reaction mixture suggested an endowise cleavage of chitosan by this enzyme.

Introduction

In the previous papers [1,2], it was reported that a bacterium belonging to the species of *Bacillus*, tentatively named as *Bacillus* R-4, produced lytic enzymes which degraded *Rhizopus* cell walls specifically. It was found that a protease and a carbohydrolase in a crude enzyme preparation brought about a partial lysis of the cell wall by each one alone and almost complete lysis by a cooperative action [2]. Of the enzymes, the protease has been isolated as a homogeneous preparation and some of the enzymatic properties were investigated [2]. The carbohydrolase appeared to be a chitosanase in nature, since a partially purified preparation showed a hydrolytic activity toward glycol chitosan, a derivative of chitosan in which ethylene glycol groups are introduced at the 6C position of glucosamine units [2].

There have been few reports concerning enzymes capable of hydrolyzing

chitosan and also participating in the lysis of fungal cell walls. Ramírez-León et al. reported that the culture filtrates of *Streptomyces* sp. were able to degrade the walls only of fungi belonging to *Mucorales* and this enzymatic complex hydrolyzed chitosan but not chitin [3]. Monaghan et al. isolated some microorganisms producing chitosanase and elucidated that this enzyme was able to lyse *Rhizopus* cell walls [4]. Hedges et al. also purified a chitosanase from the culture broth of *Myxobacter* to an ultracentrifugally and electrophoretically homogeneous state. The purified enzyme possessed a β-1,4-glucanase activity besides chitosanase and liberated glucosamine from cell wall preparations of several fungi [5]. The mode of action of chitosanase on fungal cell walls, however, still remained obscure.

The chitosanase produced by this organism, therefore, has been purified to investigate its enzymatic properties and the action patterns on chitosan as well as on *Rhizopus* cell walls. These investigations may also be expected to contribute to the elucidation of the structure of *Rhizopus* and of other fungal cell walls which have remained unclear. Some of the investigations are described in this paper.

Materials and Methods

Organism, medium and cultivation. The organism, *Bacillus* R-4, and the medium used in this study and the cultivation of the organism were described previously [1,2]. A crude enzyme preparation was obtained as described previously [1].

Preparation of Rhizopus cell walls. A sample of *Rhizopus* cell walls used as the substrate for measuring the lytic activity was prepared from pulpy mycelia of *Rhizopus delemar* according to the procedures previously described [1].

Assay of the lytic activity. Assay of the lytic activity was carried out according to the method described previously [1]. One unit was defined as an enzyme quantity which reduced 1% of the initial optical density (at 660 nm) of the *Rhizopus* cell wall suspension per 10 min.

Assay of the chitosanase activity. As described below, the enzyme hydrolyzes glycol chitosan as well as chitosan. Because of the ease in preparing the solution, chitosan was hardly dissolved in concentrations more than 0.5% owing to the viscosity; glycol chitosan was used as substrate in the assay of the chitosanase activity unless otherwise indicated. A substrate solution was prepared by dissolving 2 g of glycol chitosan in 20 ml of 0.1 M HCl with stirring. To the solution was added 40 ml of 0.2 M acetate buffer (pH 5.6) and the mixture was then adjusted to pH 5.6 with 0.1 M NaOH and made up to 100 ml with deionized water. The chitosanase activity was assayed by adding 1 ml of the enzyme solution to 1 ml of the substrate solution at 40° C. After being incubated for 10 min, sugars liberated were determined by the method of Rondle and Morgan [6]. One unit was defined as an enzyme quantity which produced 1 μmol of the sugars determined as glucosamine per min under the above conditions.

Determination of protein. Protein was estimated by measuring the absorbance at 280 nm using milk casein as a standard. The specific activity was expressed as the enzyme units per mg of protein.

Action of the enzyme on chitosan. 5 ml of the enzyme solution were incubated with 5 ml of 0.5% solution of chitosan prepared by the same method as described for the preparation of the solution of glycol chitosan at 40° C. At various intervals, the viscosity of the reaction mixture was measured by an Ostwald's viscometer and the amount of glucosamine liberated was determined by the method of Rondle and Morgan [6]. The percentage of hydrolysis was calculated as the ratio of glucosamine liberated by enzymatic digestion to that liberated by the hydrolysis with 6 M HCl at 110° C for 20 h.

Determination of molecular weight. The molecular weight of the chitosanase was estimated by gel filtration using Sephadex G-100 [7]. The purified enzyme and marker proteins dissolved in 0.5 M NaCl solution were loaded on a column of Sephadex G-100 (1.6 × 96 cm) equilibrated with 0.5 M NaCl solution. Elution was made with the same NaCl solution at a flow rate of 5.8 ml per h. The marker proteins used were cytochrome *c* (mol. wt 12 400), myoglobin (mol. wt 17 800), ovalbumin (mol. wt 45 000) and bovine albumin (mol. wt 67 000).

Disc electrophoresis. The purified enzyme (300 μg) was subjected to electrophoresis with 7.5% polyacrylamide gel in β-alanine/acetic acid buffer (pH 4.3) at 5mA for 90 min. The gel was stained with Amido black.

Chemicals. Chitosan from crab shell powder was kindly donated by Dr S. Ishiie of Godo Shyusei Co., and glycol chitosan was purchased from Wako Pure Chemicals Co. All other materials were of the highest grade commercially available.

Results

Purification of chitosanase. The enzyme was purified from the crude enzyme preparation described in Materials and Methods. Both lytic and chitosanase activities were checked throughout the purification. All procedures were carried out at 8° C unless otherwise stated.

SP-Sephadex C-50 column chromatography: The crude enzyme preparation was dialyzed against 0.003 M phosphate buffer (pH 7.6) for 50 h. The dialyzed solution was adsorbed on a column of SP-Sephadex C-50 (2.8 × 85 cm) equilibrated with 0.003 M phosphate buffer (pH 7.6). The enzyme was eluted by a linear gradient concentration of NaCl from 0 to 0.5 M at a flow rate of 30 ml per h. The effluent was fractionated into 12-ml portions.

As shown in Fig. 1, the lytic activity was separated into two fractions (Fraction I and II). Fraction I, eluted first, was the protease purified to an ultracentrifugally and electrophoretically homogeneous state as reported in a previous paper [2].

The second fraction (Fraction II) showed chitosanase activity with concomitant lytic activity. The active fractions were combined, concentrated with a collodion bag under reduced pressure and then dialyzed against 0.5 M NaCl solution.

Gel filtration on Sephadex G-100: The dialyzed solution was subjected to gel filtration on a column of Sephadex G-100 (3.6 × 150 cm) equilibrated with 0.5 M NaCl solution. Elution was made with the same NaCl solution at a flow rate of 60 ml per h and the effluent was fractionated into 18-ml portions.

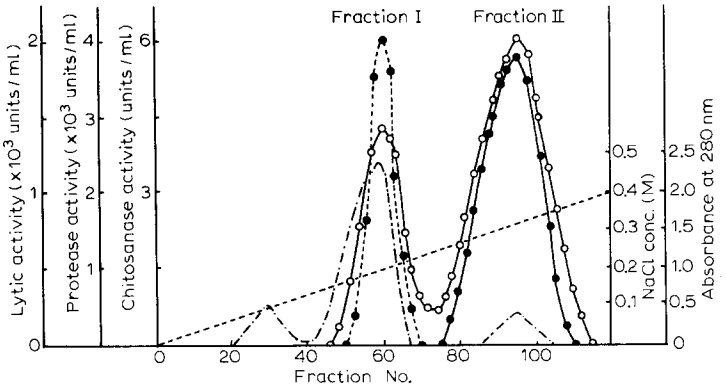

Fig. 1. Column chromatogram of crude enzyme preparation on SP-Sephadex C-50. The experimental details are described in the text. Protease activity was measured according to the method described previously [2]. ○———○, Lytic activity; ●———●, chitosanase activity; ●- - - - -●, protease activity; ——·——, absorbance at 280 nm; - - - - - -, NaCl concentration.

A typical chromatographic pattern obtained by the gel filtration is illustrated in Fig. 2. The chitosanase activity was eluted in the same fractions as those of the lytic activity. The active fractions were combined and concentrated to 10 ml with a collodion bag under reduced pressure.

Gel filtration on Sephadex G-75: The concentrated solution was purified further by gel filtration on a column of Sephadex G-75 (2.8 × 125 cm) under the same conditions as those used in the gel filtration on Sephadex G-100.

The elution pattern of the gel filtration exhibited a symmetrical protein peak accompanied by chitosanase and lytic activities in a completely constant ratio. The enzyme fractions with equal specific activity were combined and concentrated with a collodion bag under reduced pressure.

Homogeneity of the purified preparation. Homogeneity of the purified enzyme preparation was examined by ultracentrifugal sedimentation and disc

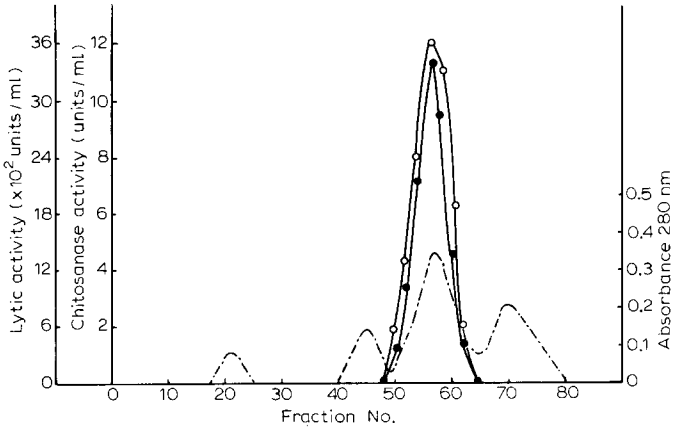

Fig. 2. Column chromatogram of chitosanase on Sephadex G-100. The experimental details are described in the text. ○———○, Lytic activity; ●———●, chitosanase activity; ——·——, absorbance at 280 nm.

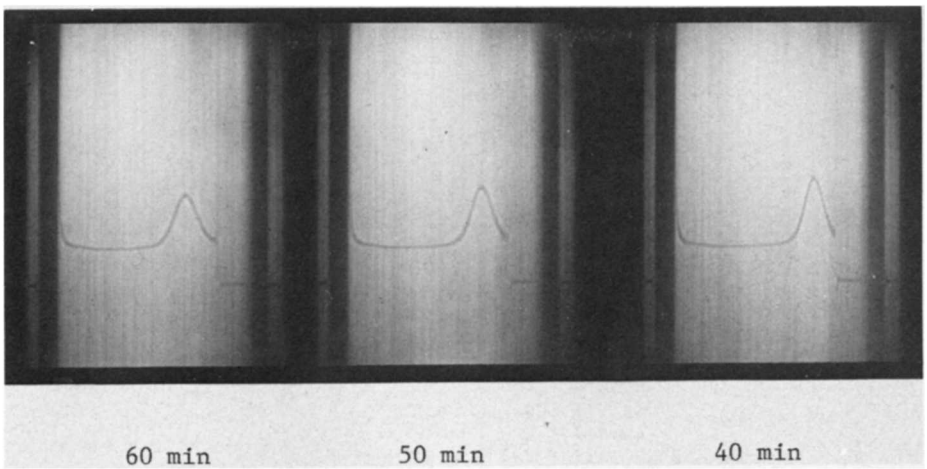

Fig. 3. Sedimentation patterns of chitosanase. Ultracentrifugation was carried out at a concentration of 0.66% purified enzyme in 0.5 M NaCl solution, with a Hitachi model UCA-1 analytical ultracentrifuge at 20° C. The photographs were taken from 40 to 60 min after reaching the maximum speed (59 600 rev./min).

electrophoresis on polyacrylamide gel, and found to be homogeneous. The results of ultracentrifugation are shown in Fig. 3.

The purification procedures are summarized in Table I. The chitosanase activity recovered in the purified preparation was 17.4% of that present in the culture filtrate with a 3560-fold increase in specific activity. This purified enzyme was used in the following experiments.

Molecular weight. The molecular weight of the chitosanase was estimated to be about 31 000 by the gel filtration on Sephadex G-100.

Isoelectric point. The isoelectric point of the chitosanase was determined by isoelectric focusing. It was estimated to be pH 8.30 from the effluent pattern shown in Fig. 4.

Ultraviolet absorption. The enzyme exhibited the maximum absorption at 278 nm and the ratio of the absorbancy at 280 nm to that at 260 nm was 1.95. The molecular extinction coefficient was calculated as being $4.96 \cdot 10^4$ $mol^{-1} \cdot cm^{-1}$ at 278 nm.

Heat stability. As shown in Fig. 5, both the chitosanase and the lytic activities were stable at temperatures below 50° C. If the enzyme was incubated at 70° C for 15 min, about 70% of the original activity still remained.

Effect of pH on the activity and the stability. Fig. 6 shows the effect of pH on the activity of the purified enzyme. Both the chitosanase and the lytic activities were most active at pH 5.6. The enzyme was stable in a range of pH 4.5 to 7.5 at 40° C for 3 h and inactivated more rapidly at alkaline pH than at acidic pH.

Effect of metal ions. The effect of various metal ions on the chitosanase activity is summarized in Table II. Among the metal ions examined, none of them activated the chitosanase, whereas Hg^{2+}, Fe^{2+}, Ni^{2+} and Zn^{2+} showed strong inhibitory effects.

TABLE I

CHITOSANASE AND LYTIC ACTIVITIES IN THE COURSE OF PURIFICATION OF THE CHITOSANASE

Fraction and step	Chitosanase activity			Lytic activity		
	Total activity (units)	Spec. act. (units/mg)	Recovery (%)	Total activity (units)	Spec. act. (units/mg)	Recovery (%)
Culture filtrate	5523.2	0.01	100.0	3.76×10^6	7	100.0
Dialyzed solution (Crude enzyme)	2435.7	0.15	44.1	1.85×10^6	112	49.2
SP-Sephadex chromatography	1693.5	31.96	30.7	4.76×10^5	8983	12.7
Sephadex G-100 chromatography	1042.9	33.93	18.9	3.11×10^5	10118	8.3
Sephadex G-75 chromatography	963.4	35.61	17.4	2.86×10^5	10571	7.6

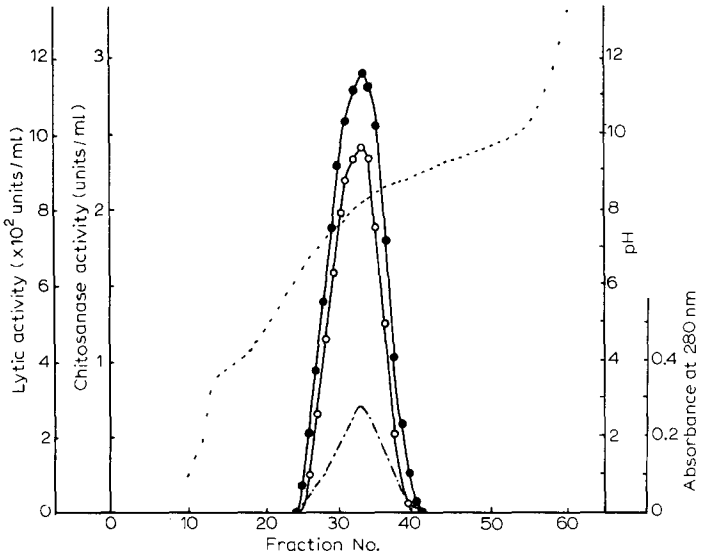

Fig. 4. Isoelectric focusing pattern of chitosanase. Isoelectric focusing in a sucrose density gradient was performed in a 110 ml capacity column using ampholytes (pH 3—10). The purified enzyme (1.32 mg) was introduced into the middle of the sucrose gradient before electrolysis was started. Electrolysis was carried out for 44 h at 4°C and the voltage was maintained at 300 V. The effluent was fractionated into 2 ml portions. o———o, Lytic activity; ●———●, chitosanase activity; - - - - -, pH; —— - ——, absorbance at 280 nm.

Effect of some reagents. The effect of some reagents on the chitosanase activity is shown in Table III. The chitosanase was activated by chelating agents, especially EDTA, and markedly inactivated by sulfhydryl reagents.

Effect of reduced glutathione and of L-cysteine on the chitosanase inhib-

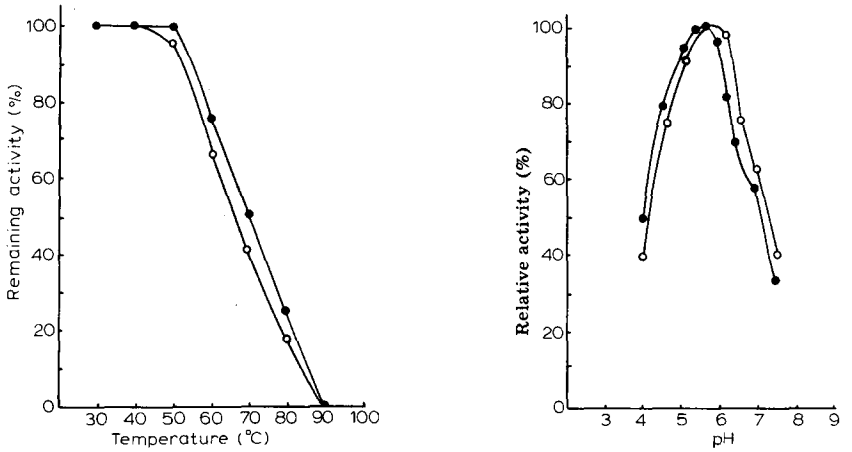

Fig. 5. Effect of temperature on stability of chitosanase. The purified enzyme, in 0.2 M acetate buffer (pH 5.6), was preincubated for 15 min at each temperature indicated and the remaining activities were measured. o———o, Lytic activity; ●———●, chitosanase activity.

Fig. 6. Effect of pH on chitosanase and lytic activities. The purified enzyme was incubated at 40°C for 10 min with glycol chitosan solution and *Rhizopus* cell wall suspension at various pH values adjusted by 0.1 M McIlvain buffer (from pH 4 to pH 7.5). o———o, Lytic activity; ●———●, chitosanase activity.

TABLE II

EFFECT OF METAL IONS ON CHITOSANASE

An enzyme solution (1 ml) of 0.2 M acetate buffer (pH 5.6) containing 10 μg chitosanase was kept at 40°C for 30 min in the presence of each metal ion indicated and the remaining activity was measured.

Metal ion	Concentration (M)	Relative activity (%)
None	1×10^{-2}	100.0
Ca^{2+}	1×10^{-2}	99.5
Ba^{2+}	1×10^{-2}	90.2
Mg^{2+}	1×10^{-2}	92.1
Mn^{2+}	1×10^{-2}	92.1
Sr^{2+}	1×10^{-2}	89.5
Mo^{2+}	1×10^{-2}	77.6
Sn^{2+}	1×10^{-2}	60.5
Co^{2+}	1×10^{-3}	100.0
Ni^{2+}	1×10^{-3}	18.8
Zn^{2+}	1×10^{-3}	37.6
Fe^{2+}	1×10^{-3}	12.2
Cr^{2+}	1×10^{-3}	0
Hg^{2+}	1×10^{-3}	3.5
Pb^{2+}	1×10^{-3}	0
Cu^{2+}	1×10^{-3}	0

ited by Hg^{2+} or sulfhydryl reagents. The effect of reduced glutathione and of L-cysteine on the chitosanase inhibited by Hg^{2+} or sulfhydryl reagents was examined. The enzyme treated with Hg^{2+}, *p*-chloromercuribenzoate or monoiodoacetate lost about 97%, 74% or 85% of the original activity, respectively, under the conditions indicated in Table IV. However, if either reduced glutathione or L-cysteine was added at a concentration 3 times higher than those of the inhibitors present in the enzyme solution, the inhibited chitosanase restored its activity almost completely in each case. It was also recognized that the addition of reduced glutathione to the native chitosanase preparation used, elevated the activity about 10%.

Mode of inhibition by monoiodoacetate and restoration by reduced glutathione. As shown in Fig. 7, the chitosanase was inactivated rapidly in an early stage of the treatment with monoiodoacetate, and more than 80% of the origi-

TABLE III

EFFECT OF SOME REAGENTS ON CHITOSANASE

An enzyme solution (1 ml) of 0.2 M acetate buffer (pH 5.6) containing 10 μg chitosanase was kept at 40°C for 30 min in the presence of each reagent indicated and the remaining activity was measured.

Reagent	Concentration (M)	Relative activity (%)
None		100.0
EDTA	1×10^{-2}	208.2
Citric acid	1×10^{-2}	117.5
p-Chloromercuri-benzoate	1×10^{-3}	12.4
Monoiodoacetate	1×10^{-2}	17.5

TABLE IV

EFFECT OF GLUTATHIONE (REDUCED) OR L-CYSTEINE ON CHITOSANASE INHIBITED BY SULFHYDRYL REAGENTS

An enzyme solution (2 ml) of 0.2 M acetate buffer (pH 5.6) containing 20 μg chitosanase was kept at 40°C for 30 min in the presence of each sulfhydryl reagent (2 mM) and the solution was divided into two portions. The chitosanase activity of one portion was immediately measured and of the other portion was measured after the addition of 1 ml of 3 mM glutathione (reduced) or L-cysteine solution.

Reagent	Relative activity (%)		
	None	Glutathione (reduced)	L-cysteine
None	100.0	111.7	100.0
$HgCl_2$	2.5	101.0	94.1
p-Chloromercuri-benzoate	25.8	94.7	102.0
Monoiodoacetate	15.3	95.3	94.3

nal activity was lost after a 5-min incubation at 40°C. The rate of inactivation, however, was relatively slow for prolonged treatment. On the other hand, if reduced glutathione was added to the inhibited chitosanase, an almost complete restoration of chitosanase activity occurred immediately, regardless of the time of the treatment with monoiodoacetate.

Effect of NaCl on solubility of the enzyme. If the chitosanase was highly concentrated or thoroughly dialyzed against deionized water, the enzyme became insoluble and was partially precipitated. Addition of NaCl, however, was found to be effective for the solubilization of chitosanase. The chitosanase was

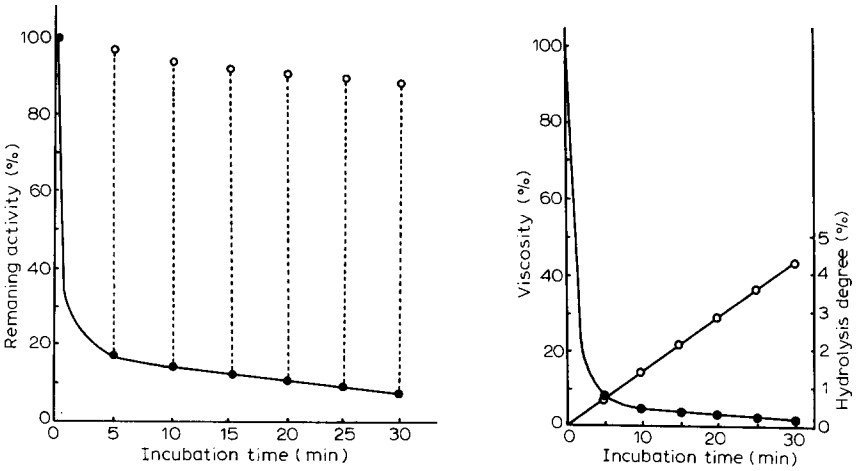

Fig. 7. Mode of inhibition of chitosanase by monoiodoacetate and restoration by reduced glutathione. The enzyme solution (2 ml) of acetate buffer (pH 5.6) containing 20 μg chitosanase was kept at 40°C in the presence of 2 mM monoiodoacetate during each period indicated and the solution was divided into two portions. The remaining activity of one portion was immediately measured and the other portion was measured after addition of 1 ml of 3 mM reduced glutathione solution. ●———●, Monoiodoacetate; ○- - - - -○, addition of reduced glutathione.

Fig. 8. Mode of action of chitosanase on chitosan. The experimental details are described in Materials and Methods. ●———●, Viscosity; ○———○, degree of hydrolysis.

completely soluble at concentrations of NaCl higher than 0.25 M but became rapidly insoluble at lower concentrations. If the chitosanase was first insolubilized in the absence of NaCl, about 80% of the insoluble enzyme could be again dissolved by addition of NaCl at a concentration of 0.5 M, but complete solubilization was not achieved by further addition of NaCl.

Substrate specificity. Activity of the purified chitosanase was examined using soluble starch, dextran, pullulan, laminaran, carboxymethyl cellulose, colloidal chitin, glycol chitin, chitosan and glycol chitosan as substrate. The enzyme hydrolyzed chitosan and glycol chitosan, but not the other glycans tested.

Mode of action on chitosan. In order to elucidate mode of action of the chitosanase, both the viscosity-decreasing and the hydrolyzing activities were examined using chitosan as substrate.

As shown in Fig. 8, a remarkable decrease in the viscosity was observed in the early stages of the reaction and the viscosity was reduced down to about 5% of the original chitosan solution after 10 min incubation when the hydrolysis of the substrate was less than 2%.

Discussion

A strain of *Bacillus sp.*, *Bacillus* R-4, has been isolated as a microorganism which lyses the cell walls of the fungi belonging to *Rhizopus* species and other related species. A carbohydrolase produced by this organism in the culture broth has been identified as a chitosanase. Since both the chitosanase and the lytic activity showed quite similar behavior in all purification procedures and in all experiments on the enzymatic properties, it has been concluded that the chitosanase is responsible for the lysis of *Rhizopus* cell walls. The organism has been shown to produce a protease which is also responsible for the lysis [2]. An abrupt decrease in the viscosity of the reaction mixture in which chitosan was used as substrate has indicated that the enzyme hydrolyzes chitosan in an endowise manner. A rapid lysis of *Rhizopus* cell walls in an early stage of the reaction as reported previously [1] may be due to this mode of action of the chitosanase.

The enzyme hydrolyzes glycol chitosan as well as chitosan, but does not hydrolyze other glycans so far examined. A chitosanase of *Myxobacter* reported by Hedges has been shown to have a β-1,4-glucanase activity in addition to a chitosanase activity [5]. It appears, therefore, that the chitosanase of *Bacillus* R-4, which does not hydrolyze carboxymethyl cellulose, differs from that of *Myxobacter*.

Many reports have already pointed out that the cell walls of *Aspergillus* and *Penicillium* which belong to *Euascomycetes* consist mainly of some kinds of glucan and chitin [8,9], and, therefore, the carbohydrolases participating in the lysis of these cell walls are principally some kinds of glucanase and chitinase [10–13]. It has been known, however, that the cell walls of *Rhizopus* and *Mucor* which belong to *Zygomycetes* are distinguishable from those of the *Euascomycetes* in that they contain chitosan as a main component besides glucan and chitin [14]. Therefore, almost all lytic enzymes so far reported are not able to degrade these cell walls. *Rhizopus* cell walls were intensively decom-

posed by the chitosanase described in the present paper which has no glucanase and chitinase activities. This fact may indicate that chitosan is present in *Rhizopus* cell walls, playing an important role in maintaining the structure of the cell wall. Thus, this chitosanase probably may be very useful for elucidating the fine structures of *Rhizopus* and other fungal cell walls which still remain obscure.

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