Studies on the Chitinolytic Enzymes of Black-koji Mold

Part VII. Degradation of Glycol Chitin and Chitin by the Chitinase System of *Aspergillus niger*

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The mode of degradation of glycol chitin and chitin by two enzyme fractions separated from *Aspergillus niger* was investigated. One of the enzyme rapidly cleaved the endo- β -glucosaminidic bonds in the polysaccharide chain, forming chitodextrin and oligosaccharides, while the other produced monosaccharide as a main product in the degradation. The successive action of the two enzymes was also examined. Intermediate products in the enzymatic degradation were surveyed using paper and column chromatography. Also, the over-all pattern of degradation of glycol chitin and chitin by the chitinase system of *Aspergillus niger* was discussed.

In a previous paper,¹⁾ the author reported that the chitinase system of a strain of *Aspergillus niger* involves two different enzymes and by their interrelated action, glycol chitin (G. Ch.) is decomposed into constituent aminosugar. In the preceding paper,²⁾ the oligosaccharidase fraction responsible for formation of aminosugar was studied by measuring N-acetyl- β -glucosaminidase activity, and the enzyme fraction which hydrolyzes β -glucosaminidic bonds in β -methyl-N-acetylglucosaminide and N,N'-diacetylchitobiose was isolated.

The chitinase system similar to that of *Aspergillus niger* has been purified from *Streptomyces griseus* by Berger & Reynolds³⁾ and from the gut of cockroach *Periplaneta americana* by Powning & Irzykiewicz,⁴⁾ al-

though in their papers the enzyme fraction which hydrolyzes both synthetic β -glucosaminide and N,N'-diacetylchitobiose is called chitobiase and acetylamidodeoxyglucosidase, respectively. Since little investigation has yet been made to elucidate the mode of action of the chitinolytic enzymes, the over-all pattern of enzymatic degradation of chitin is not clear as has been found in other polysaccharides. During investigation of this series, the author⁵⁾ has reported degradation of G. Ch. by the purified chitinase preparation which is tentatively named the liquefying chitinase. However, with oligosaccharidase fraction, the catalytic properties demonstrable over a prolonged reaction with G. Ch. and chitin have remained unstudied.

The present paper deals with the degradation of G. Ch. and chitin by the two enzyme fractions isolated from the extract of wheat-bran culture of *Aspergillus niger*, discussing

1) A. Ôtakara, This Journal, **27**, 454 (1963).

2) A. Ôtakara, *ibid.*, **28**, 745 (1964).

3) L. R. Berger and D. M. Reynolds, *Biochim. Biophys. Acta*, **29**, 522 (1958).

4) R. F. Powning and H. Irzykiewicz, *Nature*, **200**, 1128 (1963).

5) A. Ôtakara, This Journal, **26**, 30 (1962).

the over-all pattern of enzymatic degradation of chitin.

EXPERIMENTAL

Substrates.

The preparation of G. Ch. and glycol chitodextrin was made as described in the previous paper.⁵⁾

Colloidal chitin which was used as insoluble substrate was prepared by the procedure of Jeuniaux.⁶⁾ Chitin powder was dispersed in concentrated sulfuric acid solution below 10°C and the suspension was filtered through glass wool, then poured into ten volumes of cold distilled water. The reprecipitated chitin was repeatedly washed with a large volume of water by decantation and centrifugation to remove excess acid, and then dialysed against tap and distilled water. The concentration of colloidal chitin solution was estimated by weighing its dry material.

Glycol chitosan and chitosan kindly supplied by Dr. S. Okimasu were prepared by deacetylation of G. Ch. and chitin, respectively.

Enzyme Preparations.

The two different enzyme fractions, F₁ and F₂, were chromatographically separated from the extract of wheat-bran culture of *Aspergillus niger* K₁₄ as described in the previous paper.²⁾ The fraction F₁ which contained a strong chitin-liquefying activity was heated for ten minutes at 70°C to destroy the contaminating N-acetyl- β -glucosaminidase activity and was used as chitinase. The fraction F₂ possessed powerful N-acetyl- β -glucosaminidase activity with contamination of small amount of liquefying activity. This fraction was used as oligosaccharidase without further treatment. The activities of two enzyme fractions are presented in Table I. Determination of the activities was described previously.²⁾

TABLE I. ACTIVITIES OF THE ENZYME PREPARATIONS

	C. A. units/ml	G. A. units/ml	Activity ratio
F ₁	225	1.4	160
F ₂	6.6	330	0.02

Chitinase activity (C. A.) and N-acetyl- β -glucosaminidase activity (G. A.) were determined by measuring the decrease in viscosity of G. Ch. solution and the hydrolysis of β -methyl-N-acetylglucosaminide, respectively.

6) C. Jeuniaux, *Arch. Intern. Physiol. Biochim.*, **66**, 408 (1958).

Assay of the Enzymatic Degradation.

Unless otherwise indicated, the enzymatic degradation was carried out under the following condition: The reaction mixture containing 25 ml of 1% substrate solution, 15 ml of 0.2M acetate buffer solution (pH 4.0) and enzyme solution, in a total volume of 50 ml, was incubated at 40°C. At varied intervals of time, 5 and one ml aliquots of the mixture were taken to determine the increase in reducing power by a modification of the method of Willstätter & Schudel and the formation of aminosugar by the Tracey's modified method of the Morgan-Elson reaction, respectively, as described in the previous paper.⁵⁾ In heterogeneous reaction using colloidal chitin as the substrate, 7 ml aliquots of the mixture were withdrawn and boiled for 3 min and then centrifuged. Supernatant was used to determine the increase in reducing power and the formation of N-acetylglucosamine. For convenience total reducing sugars and aminosugar produced in the degradation process were expressed as N-acetylglucosamine.

Paper Chromatography.

Detection of intermediate saccharides was carried out using paper chromatography. Five ml of the reaction mixture was withdrawn and poured into 50 ml of alcohol to precipitate high molecular saccharides. After centrifugation, the supernatant was concentrated under reduced pressure and aliquot samples of the solution were chromatographed on Tôyô filter paper No. 50 with duplicate developments, using ethyl acetate-pyridine-water (120 : 50 : 40) as the solvent system. Intermediate saccharides were detected by spraying 1% potassium iodide—1% starch solution after chlorination of the paper, according to the method of Rydon and Smith.⁷⁾

RESULTS

Degradation of G. Ch. by the Two Enzyme Fractions.

As shown in Fig. 1, the mode of degradation is considerably different between the two enzyme fractions. When F₁ hydrolysed G. Ch., the increase in reducing power was remarkable in early stage without formation of detectable amount of aminosugar, whereas F₂ gradually increased reducing power accompanying with the formation of a large amount of aminosugar during degradation. After pro-

7) H. N. Rydon and P. W. G. Smith, *Nature*, **169**, 922 (1952).

longed incubation the decomposition degree of substrate measured by the increase in reducing power was greater in F_2 than in F_1 . A joint action of the two enzyme fractions increased the rate and the extent of hydrolysis, resulting in a rapid decomposition of G. Ch. to constituent aminosugar in the initial stage of incubation.

The intermediate saccharides produced in

FIG. 1. Degradation of G. Ch. by a Single and a Joint Action of F_1 and F_2 .

Reaction mixture and experimental conditions, see experimental section. Enzyme used: F_1 2 ml (C.A. 450 units), F_2 3 ml (G.A. 990 units). Open symbols: Measurement by the increase in reducing power. Solid symbols: Measurement of aminosugar formed.

FIG. 2. Paper Chromatogram of Intermediate Saccharides Produced in the Course of Degradation of G. Ch. by F_1 and F_2 .

Experimental conditions were the same as in Fig. 1. AGA: N-Acetylglucosamine, CB: N, N'-Diacylchitobiose.

digests of G. Ch. under the same condition as in Fig. 1 were surveyed using paper chromatography. As shown in Fig. 2, the reaction mixture with F_1 gave the spot tailing from the origin immediately on addition of the enzyme and its spot became distinguished as the reaction proceeded. Whereas, the spots corresponding to di- and monosaccharide did not appear in early stage of incubation, but they were detectable on prolonged incubation. On the other hand, in the reaction mixture with F_2 only the spot corresponding to monosaccharide was always remarkably found during degradation.

Since aminosugar was formed in the reaction mixture of G. Ch. with F_2 and since its formation was markedly enhanced by a joint action of F_1 and F_2 , the successive action of two enzyme fractions upon G. Ch. was then examined. As shown in Fig. 3, a rapid release of aminosugar was observed when F_2 was added after partial decomposition of G. Ch. by F_1 and the amount of aminosugar released

FIG. 3. Successive Action of F_1 and F_2 upon G. Ch.

Reaction mixture and experimental conditions were the same as those described in experimental section, except that 0.5% G. Ch. solution was used instead of 1%. The experiment was carried out using two reaction mixtures containing F_1 (C.A. 225 units). One of the reaction mixtures was used to measure a single action of F_1 and the other to measure successive action of F_1 and F_2 , by adding F_2 (G.A. 495 units) at the time indicated with arrow marks. Open and solid symbols: See Fig. 1.

after addition of F_2 was nearly equal to that of total reducing sugars measured by the increase in reducing power.

Degradation of Glycol Chitodextrin by the Two Enzyme Fractions.

The degradation of glycol chitodextrin was investigated using the same amount of enzyme as in case of G. Ch. As illustrated in Fig. 4,

FIG. 4. Degradation of Glycol Chitodextrin by a Single and a Joint Action of F_1 and F_2 .

Reaction mixture and experimental conditions, see experimental section. Enzyme used and symbols were the same as in Fig. 1.

glycol chitodextrin was also hydrolyzed by a single or a joint action of the two enzyme fractions. The decomposition to constituent aminosugar was observed in the reaction mixture with F_2 and in its single action, monosaccharide seemed to be the sole reducing sugar. However, the rate and the extent of degradation were smaller in glycol chitodextrin than in G. Ch., though the initial velocity of formation of aminosugar by F_2 was somewhat greater in the former than in the latter.

After the decomposition of glycol chitodextrin by F_1 was almost completed, the action of F_1 and F_2 was further examined as shown in Fig. 5. Neither reducing power nor aminosugar increased detectably after further addition of F_1 . Whereas, as soon as more F_2 was added, aminosugar was released rapidly and the major part of degradation products seemed to be converted into monosaccharide.

FIG. 5. Successive Action of F_1 and F_2 upon Glycol Chitodextrin.

Reaction mixture was the same as in Fig. 3, except that glycol chitodextrin was used instead of G. Ch. The experiment was carried out using three reaction mixtures containing F_1 (C. A. 225 units). One of the reaction mixtures was used to measure a single action of F_1 and two others to measure successive action of F_1 and F_2 , by adding F_1 (C. A. 225 units) and F_2 (G. A. 495 units) at the time indicated with arrow marks in Fig. a and b, respectively. Open and solid symbols: See Fig. 1.

Degradation Products of G. Ch. by the Chitinolytic Enzymes.

Four grams of G. Ch. was dissolved in 300 ml of distilled water and was adjusted to pH 4.0 with 2 N H_2SO_4 . To the solution, enzyme preparation before separating into F_1 and F_2 was added and the mixture incubated at 40°C for 100 hr. The enzyme used contained 1,700 units of chitin-liquefying activity and 3,780 units of N-acetyl- β -glucosaminidase activity. The decomposition of G. Ch. after 100 hr attained to 33.8 and 21.5% calculated by measuring reducing power and aminosugar, respectively.

After the reaction had been stopped by adding one liter of alcohol to 250 ml of the reaction mixture, the produced precipitate was collected by centrifuge and repeatedly washed with absolute alcohol and acetone. The

product was redissolved in small amount of distilled water and filtered; followed by reprecipitation with 70% acetone. The material was washed with acetone and dried in vacuo to give hygroscopic white powder in yield of 65%. This material was easily soluble in water and its solution did not give high viscosity as in G. Ch. solution. Half a gram of the material consumed 0.1 N iodine solution corresponding to 9.18 ml of 0.05 N sodium thiosulfate solution, according to the method of Willstätter and Schudel. From the amount of iodine consumed, mean degree of polymerization was calculated to be 8.9 on the basis of a molecular weight of 247 for glycol N-acetylglucosamine residue.

Supernatant resulting from treatment of the digest with alcohol was concentrated under reduced pressure below 35°C and was applied on a carbon column (Norite : Celite = 1 : 1) of 27×180 mm, which was prepared by the procedure of Zilliken et al.⁸⁾ The column was eluted successively with each 1.5 liters

of distilled water and 2.5% alcohol, and each one liter of 5, 7.5, 10 and 20% alcohol, in order to separate mono- and oligosaccharides. As shown in Fig. 6, intermediate products were surveyed using paper chromatography in the effluents with different alcohol concentrations. The spots corresponding to mono-, di- and trisaccharide were separated from the spot corresponding to higher oligosaccharide. Monosaccharide, probably glycol N-acetylglucosamine, was obtained without contamination of other products in the effluent with 2.5% alcohol. It was very hygroscopic compound with a positive Morgan-Elson reaction, but was not identified. Further investigation is now in progress to isolate and identify glycol oligosaccharides with various chain lengths.

Degradation of Chitin by the Two Enzyme Fractions.

In order to ascertain whether or not chitin is decomposed in the same fashion as in case of G. Ch., a single or a joint action of the two enzyme fractions on colloidal chitin was investigated. The data are presented in Table II. Chitin was hydrolyzed by F_1 producing more water soluble reducing sugars as compared with much less formation of N-acetyl-

FIG. 6. Survey of Intermediate Saccharides Separated by Column Chromatography from Hydrolysate of G. Ch. by the Chitinolytic Enzymes.

S: Upper, N-acetylglucosamine; lower, N,N'-diacetylchitobiose.

A: Before column chromatography.

8) F. Zilliken, G. A. Braun, C. S. Rose and P. György, *J. Am. Chem. Soc.*, **77**, 1296, (1955).

TABLE II. DEGRADATION OF CHITIN BY A SINGLE AND A JOINT ACTION OF F_1 AND F_2

Reaction time (hr.)		N-Acetylglucosamine formed (mg/5 ml)			
		4	25	50	100
F ₁	T. R. S.	0.88	1.52	1.97	2.53
	AGA	0.12	0.64	1.03	1.37
F ₂	T. R. S.	0.91	2.12	2.62	3.25
	AGA	0.90	2.03	2.76	3.55
F ₁ +F ₂	T. R. S.	1.86	2.95	3.44	3.90
	AGA	1.78	2.87	3.53	3.93

Reaction mixture and experimental conditions, see experimental section. Enzyme used was the same as in Fig. 1.

T. R. S.: Total reducing sugars measured by the increase in reducing power.

AGA: N-Acetylglucosamine measured by the Morgan-Elson reaction.

glucosamine in the earlier stage of reaction. As the reaction proceeded, N-acetylglucosamine was also remarkably formed. Whereas, during decomposition of chitin by F_2 and by its joint action with F_1 , N-acetylglucosamine was the only reducing sugar produced in soluble digest. The paper chromatography of soluble digests after incubation for 100 hr revealed that oligosaccharides were detectable together with N-acetylglucosamine in F_1 , but only N-acetylglucosamine in F_2 and in a joint action of F_1 and F_2 .

The interrelated action of two enzyme fractions was further investigated over brief reaction period at low enzyme concentration, as shown in Fig. 7. The release of N-acetyl-

FIG. 7. Successive Action of F_1 and F_2 upon Chitin.

The reaction mixture containing 5 ml of 0.5% colloidal chitin solution, 2 ml of 0.2M acetate buffer solution (pH 4.0) and enzyme solution, in a total volume of 10 ml, was incubated at 40°C. In the successive action of two enzyme fractions, the reaction mixture was preincubated in the presence of F_1 for 10 min at 40°C and the reaction was started by the addition of F_2 . Enzyme used: F_1 , C. A. 45 units; F_2 , G. A. 66 units.

glucosamine from chitin was very small in F_1 and F_2 alone, but it was fairly found when F_2 acted successively after preincubation of chitin with F_1 for 10 min, as observed with G. Ch. in the previous paper.¹⁾

Action of the Two Enzyme Fractions on Glycol Chitosan and Chitosan.

The degradation of deacetylated substrates,

glycol chitosan and chitosan, was tested under the same condition as in cases of G. Ch. and chitin. With both substrates, the increase in reducing power was not observed and both the Morgan-Elson and the Elson-Morgan reactions were negative during degradation. In addition, no hydrolysis products were detected by paper chromatography.

DISCUSSION

The over-all pattern of enzymatic degradation of aminopolysaccharides has been presented by Kent and Whitehouse,⁹⁾ assuming that it follows a pattern similar to that which has been found in other polysaccharides. On the basis of their tentative scheme, the possible pattern for the degradation of chitin and G. Ch. by the chitinase system is summarized in Fig. 8.

From the present results, it is evident that the two enzyme fractions separated from a strain of *Aspergillus niger* are different in their mode of action upon G. Ch. and chitin. One of the enzyme, F_1 , appears to cleave at random endo- β -glucosaminidic bonds of G. Ch. molecule, forming chitodextrin and oligosaccharides but not monosaccharide. The

FIG. 8. Possible Pattern for the Enzymatic Degradation of Chitin and Glycol Chitin.

9) P. W. Kent and M. W. Whitehouse, "Biochemistry of the Aminosugar" London, 1955, p. 10.

enzyme showed low specificity for glycol chitodextrin and it was separated from N-acetyl- β -glucosaminidase activity. Hence, F_1 is chitinase fraction which catalyzes a_1 and b_1 indicated in Fig. 8 and probably disaccharide is the most simple product of chitinase action. This result is quite similar to those obtained by the author for the hydrolysis of G. Ch. with the liquefying chitinase⁵⁾ purified from *Aspergillus niger* and with lysozyme.¹⁰⁾

Chitin was also hydrolyzed by F_1 according to the same fashion as in G. Ch., although considerable amount of N-acetylglucosamine was formed after prolonged reaction period. However, N-acetylglucosamine was not released in the hydrolysis of chitin with small amount of F_1 , as shown in Fig. 7. Therefore, the formation of N-acetylglucosamine may be caused by the action of the contaminating N-acetyl- β -glucosaminidase, but not by chitinase itself, because N-acetyl- β -glucosaminidase has a high affinity for N,N'-diacetylchitobiose as reported in the previous paper.²⁾ In this connection it was found³⁾ that the chitinase fraction of *Streptomyces griseus* hydrolyzed chitin into N-acetylglucosamine, probably by way of a_1 and a_2 in Fig. 8, although it did not split N,N'-diacetylchitobiose. In the hydrolysis of chitin with lysozyme,¹⁰⁾ N-acetylglucosamine was not formed even after prolonged reaction period, because lysozyme had no N-acetyl- β -glucosaminidase activity for β -methyl-N-acetylglucosaminide and N,N'-diacetylchitobiose.

On the other hand, F_2 released monosaccharide without the formation of detectable amount of oligosaccharides during degradation of G. Ch. and chitin. This finding suggests that F_2 behaves like chitinase, splitting off aminosugar from the end of polysaccharide

chain in G. Ch. and chitin molecules without random cleavage, by way of c indicated in Fig. 8. This suggestion is not consistent with the fact²⁾ that F_2 has been purified as oligosaccharidase fraction and separated from chitinase activity during enzyme purification. In addition, the author¹⁾ has previously found that aminosugar is released from G. Ch. by the interrelated action of two different enzymes. In the present investigation, the same result was also obtained in the hydrolysis of chitin demonstrable over brief reaction period at low enzyme concentration (Fig. 7). At high enzyme concentration, random cleavage of the substrates can occur in the initial stage of hydrolysis of G. Ch. and chitin by F_2 , owing to the contamination of chitin-liquefying activity. Nevertheless, no appreciable amount of oligosaccharides was formed during the hydrolysis. It is, therefore, assumed that F_2 is actually oligosaccharidase specific for low molecular saccharides and rapidly hydrolyzes not only disaccharide but also chitodextrin and oligosaccharides into monosaccharide; the enzyme catalyzes a_2 as well as b_2 in Fig. 8. The results shown in Figs. 3 and 4 suggest the possibility that F_2 attacks exo- β -glucosaminidic bonds. Comparatively little is known about the mode of action of oligosaccharidase. It is interesting in this respect that the chitobiase from almond emulsin¹¹⁾ does split chitodextrin, though that from *Streptomyces griseus*³⁾ hydrolyzes only di- and tri-saccharide, but not chitodextrin.

Recently, it has been reported by Powning and Irzykiewicz⁴⁾ that the chitinase purified from the gut of cockroach randomly hydrolyzes a series of chitin-oligosaccharides from hexaose to triose and that N-acetylglucosamine is formed with very slow rate only when

10) A. Otakara, *Kenkyū-kiyō (Hiroshima Women's Jr. College)*, **14**, 58 (1963).

11) W. Grassman, L. Zechmeister, R. Bender and G. Toth *Ber.*, **67**, 1 (1934).

triose is in relatively high concentration. Therefore, further investigation will have to be carried out on the mode of action of two enzyme fractions upon oligosaccharides with various chain lengths.

From the above findings, it seems probable that one enzyme of the chitinase system of *Aspergillus niger* is true chitinase which

randomly cleaves the polysaccharide and the other is oligosaccharidase whose action results in the complete hydrolysis of intermediate saccharides produced by chitinase action.

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