Exocellular Chitinase from a *Streptomyces* sp.

By D. M. REYNOLDS

*Department of Bacteriology, University of California, Davis,
California, U.S.A.*

SUMMARY: A number of cultures of chitin-decomposing aerobic bacteria were isolated by the enrichment technique. Chitin decomposition was followed by measurement of loss of alkali-resistant insoluble substrate. Agitated submerged cultures secured a much more rapid breakdown of chitin than static cultures. The exocellular chitinase produced by a species of *Streptomyces* in agitated submerged culture was studied. The effects of pH value and substrate concentration on enzyme activity were examined and the results used to establish a chitinase assay in terms of reducing-sugar production. The enzyme was an inducible one (adaptive). The enzyme was concentrated by ultrafiltration and lyophilization. The water-soluble reducing materials produced by the enzyme from chitin were identified as *N*-acetylglucosamine and its corresponding disaccharide, *N,N*-diacetylchitobiose.

Chitin is a tough, pliable, widely distributed natural product resembling cellulose; its fibrous structure is derived from long unbranched chains of *N*-acetylglucosamine units connected by β -1,4-glucosidic linkages. Chitin is extremely resistant to chemical reagents; strong mineral acids are required to dissolve it.

Most of the published reports of microbial chitin decomposition are concerned with taxonomic and physiological studies rather than with the enzymic aspects of the process. Benecke (1905), the first worker to isolate and study a chitin-decomposing microbe, stated that his '*Bacillus chitinovor*' (a non-sporeformer) must possess a chitinase, but found no evidence of saccharidic products of chitinase activity. Folpmers (1921) suggested the existence of a water-soluble cell-free chitinase from the fact that his cultures of chitin-decomposing Eubacteriales and actinomycetes produced clear zones in agar prepared with precipitated chitin. ZoBell & Rittenberg (1938), from experiments with marine chitin-decomposing bacteria, suggested that chitin breakdown might result from either a cleavage of the glycosidic linkages or a splitting-off of the aminoacetyl group.

Karrer & Hofmann (1929) used a snail gut enzyme to secure up to 85 % conversion of chitin into *N*-acetylglucosamine, thus helping to establish that compound as the unit building block of the polysaccharide. No other enzymic breakdown products of chitin were identified. Grassmann & Rubenbauer (1931) found that 'Luizym', a commercial product prepared from dried aqueous extracts of *Aspergillus niger*, split chitin into iodometrically detected reducing material of undetermined nature. Zechmeister, Tóth & Balint (1938) and Zechmeister & Tóth (1939) demonstrated that almond emulsin could be chromatographically separated into a β -glucosidase, an α -galactosidase and a chitinase. The latter was further separated into a polysaccharidase and a

second enzyme which preferentially hydrolysed the disaccharide building block of chitin, *N,N*-diacetylchitobiose. Jeuniaux (1950, 1951) found that chitin-decomposing Eubacteriales from the snail gut and chitinoclastic soil actinomycetes produced exocellular chitinases.

EXPERIMENTAL

Preparation of chitin substrate

To secure a substrate for culture media, a supply of partly purified chitin was prepared according to the usual acid and alkali method, with the realization that a certain amount of denaturation was unavoidable. Shell fragments (< 2 mm.) of the Dungeness crab, *Cancer magister*, were held in a mechanically stirred suspension of dilute HCl and maintained at pH 2.0–3.0 by repeated addition of acid for 3 days to remove calcium salts. After washing, the material was suspended in 4 vol. of 5% (w/v) KOH and brought to 100° in an autoclave for 3 hr., with intermittent mechanical stirring, to render the non-chitinous constituents soluble. The alkali treatment was repeated, the chitin washed and then dried at 70°. After grinding through a 0.5 mm. mesh screen Wiley mill, the acid and alkali treatments were repeated, the oven-dried chitin Soxhlet extracted with 95% ethanol, and the dry powder ground in a ball mill. The yield was 17%.

A purified lot of chitin for use as a substrate for the assay of chitinase was prepared by extracting the above material with 2.5% (v/v) acetic acid to remove any chitosan possibly formed by the KOH treatment, followed by 95% ethanol extraction. The product was almost white in colour and had an ash content of 1.25%. Microchemical analysis gave the following results: calculated—C, 47.3; H, 6.4; N, 6.89; —COCH₃, 22.17%; found—C, 43.1; H, 7.0; N, 6.87; —COCH₃, 19.2%.

Colloidal precipitated chitin was prepared roughly according to the procedure of Bucherer (1935) and preserved in the frozen state.

Isolation and testing of chitin-decomposing bacteria

Enrichment cultures were set up using a mineral salt medium (K₂HPO₄, 0.7 g.; KH₂PO₄, 0.3 g.; MgSO₄·5H₂O, 0.5 g.; FeSO₄·7H₂O, 0.01 g.; ZnSO₄, 0.001 g.; distilled H₂O, 1 l.; pH adjusted to 7.0) to which powdered chitin (0.25%, w/v) was added as a sole source of C and N. The medium was dispensed in 5 mm. layers in Erlenmeyer flasks; separate flasks of each medium were inoculated with dilutions of eight garden and forest soils. The flasks were incubated both statically and on a rotary table shaker (New Brunswick Scientific Co.) which described a circle of 2.5 cm. diam. and ran at 325 r.p.m. All culture incubation described in this paper was at 28°.

To isolate pure cultures of chitin-decomposing microbes, we plated out the above enrichments after 2 weeks on to neutral mineral salts agar containing sufficient colloidal precipitated chitin to render it cloudy. Bacteria were isolated and purified from 41 colonies which formed clear zones in the chitin. The above enrichment procedure, using chitin as a sole C and N source,

admittedly selected against nutritionally fastidious chitin-decomposing microbes.

These 41 isolates were tested for ability to decompose chitin in agitated submerged culture, following approximately the method of Reese (1947) for aerobic cellulose decomposition studies. Portions (250 mg.) of powdered chitin were weighed into 250 ml. Erlenmeyer flasks which were uniformly inoculated and incubated on the rotary shaker for 15 days. Sufficient 5 N-KOH was then added to make the KOH concentration 0.5 N, all growth submerged in the alkaline solution, and the flasks autoclaved at 121° for 15 min. The alkali-insoluble residue was collected in a tared 30 ml. Sela semi-porous crucible (No. 3001), washed with water, and dried at 105° for 16 hr. The cooled crucibles were weighed, ignited for 8 hr. at 650°, and reweighed for ash. An experiment with uninoculated flasks showed that $100 \pm 1\%$ of the chitin was recovered by this method. The decomposition of organic matter was calculated as

$$\% \text{ loss of organic matter} = \frac{[\text{post-alkali residue}] - [\text{culture ash}]}{[\text{original chitin, water-free}] - [\text{culture ash}]} \times 100.$$

Since the bacteria formed a certain amount of alkali-resistant polysaccharide during growth, the actual percentage of chitin loss was always somewhat greater than indicated by the data.

When subjected to the above screening procedure, all of the zone-producing organisms showed some ability to decompose chitin in aerated submerged culture. The amount of chitin decomposed ranged between 10 and 97 % of the starting material. In a number of cases, the amount of decomposition strikingly increased when yeast extract (0.05 %, w/v) was added to the medium, e.g. in one instance from 17 to 90 % chitin loss over 15 days. The study of such nutritional aspects of chitin decomposition will be reported later. For the purpose of this investigation, in which a culture fluid with chitinase activity was sought, those cultures which could actively decompose chitin with that compound as a sole source of C and N were chosen for further study. The avoidance of complex organic media would render subsequent enzyme concentration and purification less difficult.

The speed of chitin breakdown with the three most active organisms in the above experiment (two *Streptomyces* spp. and a true bacterium) was ascertained by inoculating each organism into eight replicate flasks of the same medium, and removing pairs at intervals for alkali treatment. The results (Fig. 1) showed that a rapid chitin breakdown took place in submerged cultures of all three organisms. With static incubation of 5 mm. deep layers of media, the amount of chitin breakdown after a month was only a small fraction of that secured with shaken flasks of these aerobic cultures in 5 days.

Demonstration of a cell-free microbial chitinase

The above data were used as a guide to harvesting identical submerged cultures of the three organisms for an examination for chitinase activity in the culture fluids. After 4 days' growth with the *Streptomyces* spp. (C-10 and C-14) and 8 days for the bacterium (C-25), the cell material was centrifuged

off and the supernatants preserved by the addition of sufficient 1% (w/v) aqueous thiomersalate to produce a concentration of 0.01% (w/v). The three solutions were each divided into equal parts which were adjusted, respectively, to pH's 5.0 and 8.0.

A test for chitinase activity was made on these solutions by dispensing 50 mg. of the ball-milled highly purified chitin into 50 ml. Erlenmeyer flasks, adding 10 ml. of the cell-free fluid so as to produce a homogeneous suspension, and incubating the tinfoil-capped flasks for 48 hr. at 28° on a slower (120 r.p.m.) table shaker running in a 2.5 cm. diam. circle. Two controls were used: (i) flasks with the chitin in mineral salt solution; (ii) cell-free culture fluid without chitin; both controls contained thiomersalate. After incubation, the enzyme was inactivated by immersing the flasks for 5 min. in a 100° water-bath. Approximately 3 ml. of the flask suspensions were centrifuged at 18,000 *g* to remove residual chitin. Samples (1.0 ml.) of the supernatant solutions were added to pairs of optically uniform Pyrex test tubes for reducing-sugar assay by the dinitrosalicylic acid (DNS) method of Sumner & Somers (1949) with the assay adjusted to the range 0.05–0.9 mg. glucose/ml. While both thiomersalate and phosphate salts can interfere with the DNS assay, this was not observed with the concentration of these substances present in the culture supernatants.

Table 1 summarizes the results of the above test at pH 8.0 for chitinase activity in the three organisms. The reducing-sugar found in flasks containing cell-free culture fluid was appreciably higher than that found in flasks containing fluid alone. In this and all subsequent experiments, the control flasks with cell-free fluid alone contained either no or at most a trace of reducing substance detectable by the procedure used. Likewise, the controls with chitin incubated with mineral salts solution were consistently negative for reducing sugars. *Streptomyces* sp. C-10 was selected for further enzyme work.

Table 1. *Chitinase activity of cell-free culture fluids from three submerged cultures of chitin-decomposing bacteria*

The figures represent averages of two flasks, each assayed in duplicate.

Culture	Solution assayed	Reducing-sugar as glucose (mg./ml.)
C-10	Cell-free culture fluid	< 0.05
	Cell-free culture fluid + chitin	0.38
C-14	Cell-free culture fluid	< 0.05
	Cell-free culture fluid + chitin	0.26
C-25	Cell-free culture fluid	0.06
	Cell-free culture fluid + chitin	0.15
None	Chitin + min. salt solution	< 0.05

The observed chitinase activity was shown to be due to a cell-free enzyme (or enzymes) by filter sterilization of active centrifuged C-10 culture fluid with Morton Ultra Fine porosity sintered glass filters. Solutions so sterilized had a chitinase activity; a sterility test showed that no contamination took place before or during the period of enzyme activity.

Some properties of the enzyme

pH range. With thiomersalate-treated cell-free culture fluid, enzyme solutions containing M/15 phosphate were prepared over the pH range 4.5–8.3; the range was extended to pH 3.0 with HCl. Additional buffer capacity to that of the phosphate was presumably provided by dissolved protein. Duplicate 10 ml. samples were assayed as above in 50 ml. flasks containing 30 mg. chitin incubated on the shaker at 28° for 72 hr. The pH change in all but three of the nineteen different values of enzyme solution did not exceed 0.2 unit during the experiment; in three, it did not exceed 0.4 unit. The assay results (Fig. 2) indicated a pH optimum *c.* pH 6.5–7.5, so that subsequent assays were run at pH 7.0.

Fig. 1. Aerobic decomposition of chitin by three soil organisms in agitated submerged culture; C-10 and C-14 *Streptomyces* spp., C-25 a bacterium.

Fig. 2. The effect of pH on the activity of *Streptomyces* sp. C-10 chitinase.

Effect of substrate concentration. Chitin was distributed into pairs of Erlenmeyer flasks (75 mg./flask) and cell-free culture fluid added as follows: 10 ml./50 ml. flask; 25 ml./125 ml. flask; 50 ml./250 ml. flask; 75 ml./300 ml. flask. The flasks thus containing 7.5, 3.0, 1.5 and 1.0 mg. chitin/ml. solution were then assayed with controls, using 28° and 48 hr. incubation. In all the flasks, a large portion of the insoluble chitin was still visible after the assay. The results of the experiment (Fig. 3) led to the selection of 3 mg. chitin/ml. (actually 30 mg./10 ml.) for all subsequent assays. Since chitin is insoluble in water, one can readily establish that this substrate has not become exhausted during the course of a chitinase assay. Kinetic experiments have also shown that 3.0 mg. chitin/ml. is an adequate substrate concentration for assay work.

Improvements in the assay were later made when it was found that the enzyme had a temperature optimum at 37°. While at 20 and 28° rotary agitation increased the amount of reducing-sugar production, at 37° equal amounts of product were formed in both swirled and static flasks, thus rendering agitation unnecessary.

Enzyme induction by chitin. *Streptomyces* sp. C-10 was grown in the neutral mineral salt medium in agitated submerged culture with chitin (0.5 %, w/v), *N*-acetylglucosamine (0.3 %, w/v), or glucose (0.1 %, w/v) + asparagine (0.05 %, w/v).

w/v). The *N*-acetylglucosamine was sterilized by filtration. With the two monosaccharide media, growth of the actinomycete was moderate in amount; with the chitin medium, heavy. After given periods of time, the supernatants from pairs of duplicate flasks were assayed for chitinase activity. The results (Table 2) showed that only with the organisms grown in chitin was there evidence of formation of reducing-sugars from chitin by the cell-free culture fluids; the enzyme thus exhibited an induced (adaptive) behaviour.

Two β -1,4-linked polymers of glucose configuration sugars (cellulose and cellobiose) were not hydrolysed by the enzyme; it appears that if compounds of this type are to be cleaved by the enzyme, they must possess the amino-acetyl group.

Table 2. *Effect of substrate on chitinase production by Streptomyces* sp. C-10

Compounds added to basal mineral salt medium	Age of culture (days)	Enzyme activity of cell- free culture fluid		Reducing-sugar, as glucose, produced by chitinase (mg./ml.) (2) - (1)
		Alone	With chitin	
		Reducing-sugar formed, as glucose (mg./ml.)		
		(1)	(2)	
Glucose+Asparagine	5	0.77	0.78	0.1
	6	0.35	0.35	0.0
<i>N</i> -acetylglucosamine	3	0.95	0.95	0.0
	5	< 0.05	< 0.05	0.0
Chitin	5	< 0.05	0.28	0.28

Stability of the enzyme. C-10 chitinase activity is very stable when the solutions are treated with 0.01 % (w/v) thiomersalate, brought to pH 7 and stored at 4°. A sample of cell-free fluid stored under these conditions for 4 months showed only about 25 % loss of activity.

Production of the enzyme in mass culture. To determine when maximum chitinase activity was reached, 2 l. Erlenmeyer flasks containing 1 l. of medium were inoculated at intervals over a period of 2 weeks with 50 ml. of a 3-day-old submerged C-10 culture and incubated on the shaker at 250 r.p.m. The flasks, ranging in age from 2 to 14 days, were simultaneously harvested and the culture supernatants assayed in the usual way. The results (Fig. 4) showed that peak chitinase activity was reached at 6 days incubation and fell off thereafter. The peak of activity coincided approximately with the time at which the last remaining chitin disappeared from the bottom of the flask.

A similar time study on chitinase formation was conducted with cultures in which the chitin neutral mineral salts medium received separately different nitrogenous supplements: Difco yeast extract (0.2 or 0.05 %), asparagine (0.1 %), or a mixture of NaNO₃ and NH₄NO₃ (0.07 and 0.03 %, w/v). The basal chitin mineral salt medium gave higher peaks of chitinase activity than any of the media which received these nitrogenous supplements. Earlier experiments on chitin break-down by organisms which could grow with chitin as a sole source of carbon and nitrogen had shown that a number of these bacteria were much more effective in decomposing the polysaccharide when yeast extract was

added to the medium, but *Streptomyces* sp. C-10 did not respond in this manner to yeast extract supplementation. It was therefore not surprising that this organism produced no increase in exocellular chitinase when yeast extract was added.

Concentration of the enzyme by ultrafiltration and lyophilization

A preliminary concentration of enzyme was made by means of nitro-cellulose ultrafilters prepared after the procedure of Kabat & Mayer (1948); a glass mantle surrounding the filter thimble allowed a greater degree of con-

Fig. 3. The effect of substrate concentration on reducing sugar production by *Streptomyces* sp. C-10 chitinase.

Fig. 4. Production of exocellular chitinase by *Streptomyces* sp. C-10 in agitated submerged culture with a chitin-mineral salts medium at different times of growth.

centration than in their technique. Active culture fluid (10 l.) was chilled to 5°, clarified with a Sharples supercentrifuge, treated with thiomersalate, adjusted to pH 7.0 and concentrated to 100 ml. (100-fold) with an ultrafilter prepared with a 5% (w/v) cellulose nitrate solution. Controlled chitinase assays on the original solution (0.32 mg. reducing-sugar/ml.), the solution passed by the ultrafilter (<0.05 mg./ml.) and the solution retained by the ultrafilter (14.0 mg./ml.) indicated that a marked concentration of the enzyme was secured by ultrafiltration. Further concentration of the ultrafilter-retained solution to the dry state without any loss of activity was made by freeze-drying.

A brown precipitate appearing on the surface of the ultrafilters was enzymically inactive and was subsequently separated from all ultrafilter concentrates by the Sorvall SS-1 centrifuge at 18,000 g.

Identity of chromatographically detectable enzyme products

For chromatographic detection of reducing sugars, the phenylenediamine spray reagent of Chargaff, Levine & Green (1948), modified by Mr F. Stadtman (1953, personal communication) by the substitution of oxalic acid for HCl was used; the reagent is prepared by dissolving 0.2% (w/v) oxalic acid in approximately 95% ethanol. *o*-Phenylenediamine (instead of the *meta* isomer used by Chargaff *et al.*) was used, since the former on heating at 100° for 15 min. gave more distinguishable colours in daylight. A preliminary experiment showed that *N*-acetylglucosamine was completely hydrolysed to glucosamine

when it was dissolved at 5% (w/v) in N-HCl and heated to 100° for 5 hr. in a sealed test tube.

A solution of the unknown reducing material produced by the enzyme was obtained by allowing 50 ml. active cell-free culture fluid to react with 500 mg. of highly purified ball-milled chitin in a 500 ml. Erlenmeyer flask at 28° on the 120 r.p.m. shaker. After 7 days, a considerable amount of insoluble chitin remained in the flask. The suspension was heat inactivated, centrifuged and the supernatant passed through a small de-ionizing column filled with a mixture of cationic and anionic exchange resins. The solution was then concentrated to about 0.5 ml. (100-fold) by evaporation *in vacuo* at 70°. A similar control concentrate was made of an identical enzyme solution which had not accumulated reducing material by incubation with chitin. Whatman No. 1 filter-paper was set up with 10 μ l. spots of the following materials: glucose (1%, w/v), glucosamine : HCl (5%, w/v), *N*-acetylglucosamine, both untreated and acid-hydrolysed (5%, w/v), the above enzyme concentrates, both control and chitin-reacted, and the latter concentrate acid-hydrolysed as above. Development of the descending 36 hr. phenol/water/ammonia chromatogram showed that the control enzyme preparation contained no detectable reducing compounds, whereas the concentrate of the enzyme previously reacted with chitin showed two spots, one with the same R_F and colour as secured with *N*-acetylglucosamine and a second more rapidly moving unidentified compound. The identity of the slower enzyme product with *N*-acetylglucosamine was confirmed by the fact that the HCl-hydrolysed enzyme products gave a single spot with the same R_F and colour as glucosamine, which is under these conditions the hydrolysis product of *N*-acetylglucosamine.

Since the faster spot was also converted to glucosamine on acid hydrolysis, it seemed likely that it might be *N,N*-diacetylchitobiose. We secured 0.75 g. of crystalline octa-acetylchitobiose which had been prepared from chitin after Bergmann, Zervas & Silberkweit (1931). It had an m.p. of 306–308° (decomp.) and $[\alpha]_D^{25} + 56.5 \pm 0.4$ (1%, HOAc); these values are not in accord with those (m.p. 289° and $[\alpha]_D^{25} + 50.3^\circ$) secured by Bergmann *et al.*, but are in agreement with those obtained for the same octa-acetate by Zechmeister & Tóth (1931): 305° and $[\alpha]_D^{25} + 55.3^\circ$. The O-acetyl groups were selectively hydrolysed from the octa-acetate by means of the Isbell barium methylate procedure described by Bates *et al.* (1942), yielding *N,N*-diacetylchitobiose.

The above described phenol/water/ammonia chromatogram was repeated with the addition of *N,N*-diacetylchitobiose and chitobiose octa-acetate. Both the R_F values and the colours obtained with spots of the respective substances (Fig. 5) confirmed the earlier experiment and indicated that the previously unidentified enzymic product was *N,N*-diacetylchitobiose. The chromatogram was repeated with a collidine/water system with the same net results: two enzymic products were evident, with the R_F values and spot colours of *N*-acetylglucosamine and *N,N*-diacetylchitobiose. With collidine/water the biose moved more slowly than the *N*-acetylglucosamine. In none of the chromatograms was there evidence that either glucosamine or glucose were produced from chitin by this enzyme. The product solution was examined with

a two-dimensional chromatogram using the solvent system phenol/water/ammonia followed by butanol/ethanol/water/ammonia (45 : 5 : 49 : 1). This experiment revealed no more than the two products already identified.

Fig. 5. Phenol/water/ammonia paper chromatogram of the water-soluble reducing products resulting from the hydrolysis of chitin by *Streptomyces* sp. C-10 chitinase. Spots developed by phenylenediamine and oxalic acid reagent. Colours of spots in daylight: [·] = blue; [/] = brownish green; [\] = reddish brown; [—] = greenish brown; [—] = greenish blue.

DISCUSSION

From our data it appears that enzymic chitin breakdown may follow the pattern of a number of other polysaccharides in that one or more enzymes degrade the compound to a biose stage which is then split by another enzyme. Supporting evidence of this lies in the fact that another similarly prepared chitinase product concentrate on chromatographic examination showed only a *N,N*-diacetylchitobiose spot; no monose was detected. Other polysaccharidases which accumulate bioeses include the classical amylose → maltose phenomenon, the cellulose → cellobiose mechanism shown by Levinson, Mandels & Reese (1951) and by Whistler & Smart (1953), the xylan → xylobiose pathway (Sørensen, 1952) and the pectic acid → digalacturonic acid route of Dr A. L. Demain and Dr H. J. Phaff (1954, personal communication).

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