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Chitinase in some Basidiomycetes

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The decomposition of chitin by bacteria has been the subject of sporadic interest, but its breakdown by fungi has attracted little interest. Chitin may be assumed to be one of the polysaccharides that are of quantitative importance in the complex series of microbiological syntheses and analyses that occur in any natural soil. Since, like cellulose, chitin is an insoluble polymer of low hydration built up of closely apposed oriented chains, it is likely to be relatively resistant to enzymic breakdown (Tracey, 1953). Most of the fungi contain chitin, and it occurs also in most groups of the soil fauna save perhaps the Protozoa. As a relatively abundant material, probably only slowly broken down enzymically, it may prove of importance as a constituent of the total organic matter of the soil and, even more, of the non-living fraction. Chitin is normally estimated by total hydrolysis followed by determination of glucosamine and sometimes of acetic acid. The method is not specific even if preceded by extensive purification of the material (a process that often includes alkali treatment with risk of deacetylation). Glucosamine is broken down to a variable extent during acid hydrolysis and in crude preparations may arise from substances other than chitin. The use of enzymic hydrolysis should serve as a useful check on chemical methods, for it has the advantage that the end product is acetylglucosamine and any doubt about the acetylated form of the original material is removed. The present work was done in the hope of locating a useful source of chitinase for analytical work (Tracey, 1955). Liquids of considerable chitinase activity have been found to be produced by a number of species of *Lycoperdon*. The fungi are abundant at the beginning of autumn and the enzyme solutions obtained from them are stable for considerable periods.

METHODS

Fungal extracts. Basidiomycete fructifications were minced, and the liquid was then expressed through cloth and centrifuged. Some fungi gave on mincing a gelatinous mass from which it was impossible to express an extract by hand. With these minces a cloth-lined basket centrifuge gave an extract with little trouble. Many *Lycoperdon* species, if harvested at a suitable stage of maturity, contain free liquid which usually accounts for 70–75% of the fresh weight. These fructifications were torn open and spun on the basket centrifuge without mincing. A further extract of considerably higher solid content could be got by mincing the residue from the first treatment.

Chitosan. This is a product of variable composition prepared by the partial deacetylation of chitin (see, for example, Darmon & Rudall, 1950). Lobster (*Homarus*) chitin was heated with 40% (w/v) NaOH solution in a flask fitted with a Bunsen valve in an oven at 105° until the material was soluble in dilute acetic acid. Excess of NaOH solution was decanted after centrifuging, the chitosan dissolved in dilute acetic acid and dialysed against distilled water to give a solution of the salt, chitosan acetate.

Chitin. Cuttlefish (*Sepia*) 'bones' were soaked in dil. HCl until all $CaCO_3$ had been removed. The chitinous laminae were separated from sand and dispersed in cold conc. HCl. As soon as the solution appeared substantially complete the liquid was centrifuged and the supernatant poured into a large volume of distilled water with mechanical stirring. The precipitated chitin was washed repeatedly by decantation and residual material of low molecular weight removed by dialysis against distilled water. The chitin was stored as

a suspension in water (1 g. N/l.) with added toluene as an antiseptic. It gave the theoretical yield of acetylglucosamine on enzymic hydrolysis and of glucosamine and acetic acid on acid hydrolysis.

Estimation of acetylglucosamine and glucosamine. Modifications of the methods of Morgan & Elson (1934) and Elson & Morgan (1933) were used (Tracey, 1955). These methods have an accuracy of ±3% over the range 0·5–10 μg. of glucosamine or acetylglucosamine N in the sample. The colorimetric method for glucosamine is positive for galactosamine, chondrosin, heparosin and hyalobiuronic acid and would be expected to be so for chitobiose and other oligosaccharides containing a terminal glucosamine unit with free reducing group. The colorimetric method for acetylglucosamine is positive for acetylgalactosamine (one-fifth the colour yield), blood-group mucoids (Aminoff, Morgan & Watkins, 1952); it is not given by *NN'*-diacetylchitobiose, 4-β-galactopyranosyl-*N*-acetylglucosamine or 3:4:6-trimethyl-*N*-acetylglucosamine (Kuhn, Gauhe & Baer, 1954; Zilliken, Braun, Rose & György, 1955; Zilliken, Smith, Tomarelli & György, 1955). Other substances that may interfere are unlikely to be released from chitin of the purity used in these experiments.

Viscometric assay. The viscosity of chitosan solutions is markedly reduced by added salts. The effect of varying concentrations of NaCl on the viscosity of a dialysed solution of chitosan acetate is illustrated in Fig. 1. In order to

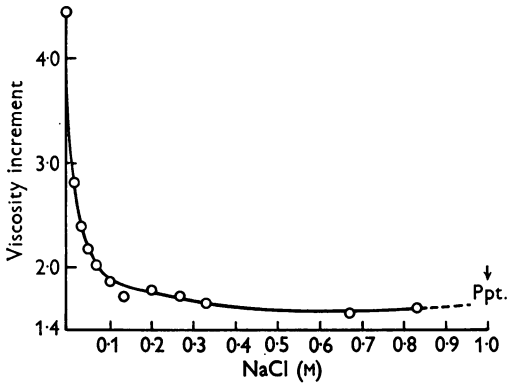

Fig. 1. Effect of increasing concentrations of NaCl on viscosity of chitosan acetate (concentration 4·05 g./l.). A fine precipitate began to form at NaCl concentrations between 0·8 and 0·9 M; it appeared complete at about 1·0 M.

reduce interference by salts present in unknown concentration in the solutions to be assayed, NaCl was added to the stock solution of chitosan acetate to give a final concentration of 0·3 M-NaCl and 11·4 g. of chitosan acetate/l. Since in the viscometric chitinase assays the solution was diluted by the addition of one volume of buffer and one volume of test solution it was desirable to use a buffer solution with added salt. The buffer was prepared by adding 19 g. of hydrated sodium acetate, 23·4 g. of NaCl and 3·6 g. of acetic acid to 800 ml. of water, adjusting the pH to 5·0 if necessary, and diluting to 1 l. Its molarity in terms of Na was 0·56. Thus the final mixture of substrate, buffer and test solution had a minimal Na content of about 0·3 M and any salts in the test solution were unlikely to affect the viscosity of the mixture appreciably unless there was visual evidence of precipitation.

Chitosan acetate NaCl solution (1 ml.) was mixed in a small beaker with 1 ml. of buffer. At a known time 1 ml. of the test solution was added to the beaker, the contents were rapidly mixed by swirling and 2·5 ml. of the mixture were immediately pipetted into a U-tube viscometer vertically supported in a water bath at either 25° or 30°. The flow time was determined at frequent known intervals from the time of mixing. Blank determinations, in which water replaced the test solution and in which water alone was used, were made.

Using the time for water alone the relative viscosity of the chitosan blank and the relative viscosity of the enzyme–substrate mixture at each time of observation were calculated. A plot of viscosity increment (relative viscosity – 1) against time was then used to determine the time required for the loss of half the viscosity increment (or, if more convenient, some other fraction). A convenient unit of activity was that amount of enzyme required to halve the viscosity increment in 100 min.

Assay by chitin hydrolysis. A dialysed suspension of *Sepia* chitin (1 ml.) and 0·2 M sodium acetate buffer pH 5·0 (2 ml.) were incubated, together with the test solution and sufficient water to give a final volume of 5 ml. in a stoppered tube at 35°. Toluene was used as an antiseptic. Blanks of substrate alone and test solution alone were also set up. At intervals samples were withdrawn and the total amount of acetylglucosamine in solution was determined.

RESULTS

Viscometric assay

Many fungal extracts reduced the viscosity of chitosan acetate solutions at pH 5. Typical observations on a commercial enzyme solution (Enzyme 19AP, Rohm and Haas, Philadelphia, described as containing cellulase and hemicellulases) are illustrated in Fig. 2. The observations are capable of quantitative interpretation in terms of enzyme concentration. Results for the comparative activities of a number of fungal extracts are listed in Table 1.

The effect of a number of sugars on the rate of hydrolysis of chitosan was measured. At a final concentration of 0·25 M (concentration in viscometer fluid) glucose, sucrose, galactose and glucosamine hydrochloride reduced the apparent activity of a *Lycoperdon giganteum* extract to about 75% of that found in their absence. The same concentration of acetylglucosamine reduced the apparent activity to 55%. This greater effect of acetylglucosamine may perhaps be due to a specific inhibition of the enzyme by one of the products of its action. Prolonged hydrolysis of a sample of the chitosan by an active *L. giganteum* extract resulted in the formation of acetylglucosamine equivalent to only 10·5% of the total N content. Since acetylglucosamine gives a colour in the method used for

determination of glucosamine (16% of that of an equimolar amount of glucosamine) it is difficult to estimate very small quantities of glucosamine in the presence of large amounts of the acetylated form. The amount of glucosamine present after prolonged enzymic hydrolysis of the chitosan was certainly less than 10% of the acetylglucosamine and was probably nil. The chitosan sample contained 2·35 N atoms/acetyl group, indicating that 42% of the glucosamine residues were acetylated.

The utility of the method for following enzyme production was demonstrated by measuring the progress of chitinase production by a culture of *Streptomyces albidoflavus* grown in a mineral salt medium containing 5 g. of *Sepia* chitin/l. The pH of the medium was 7·2 and incubation was at 25° (Fig. 3).

Assay by chitin hydrolysis

In the method for determination of acetylglucosamine, colour formation was depressed by citrate (0·05M), concentrated salt solutions [e.g. Na_2SO_4 in a concentration of 7·5% (w/v) depressed colour formation by 33%] and formate. Crude mycelial extracts may contain other substances effective in lesser amounts. An extract of *Penicillium griseo-*

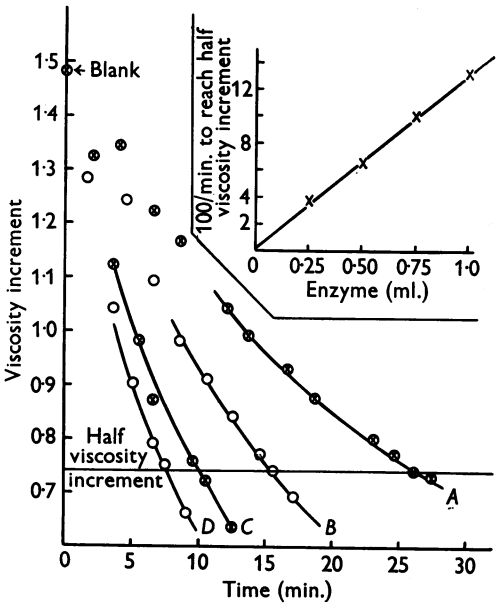

Fig. 2. Estimation of chitinase in enzyme 19AP by viscometric method. Chitosan acetate substrate, pH 5·0, 25°. Plot of viscosity reduction against time that has elapsed since mixing: *A*, 0·25 ml. of enzyme solution (5 g. of enzyme preparation in 100 ml. of water, dialysed and centrifuged); *B*, 0·5 ml.; *C*, 0·75 ml.; *D*, 1·0 ml.

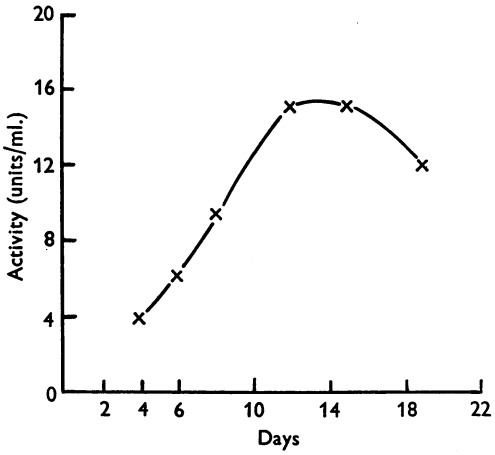

Fig. 3. Chitinase production during the growth of *Streptomyces albidoflavus* culture. Viscometric assay.

Table 1. *Chitinase activities of fungal and snail extracts (viscometric assay)*

	Units/ml.	Units/g. of dry matter
Coprinus comatus (autolysis fluid from mature caps)	20–80	—
Phallus impudicus (deliquescent hymenium)	30	—
Fistulina hepatica	2	—
Bovista plumbea	25	—
Lycoperdon depressum	100–250	—
L. giganteum	200–1000	5×10^4 (5×10^5 dialysed)
L. pyriforme	200–1000	10^5 (5×10^5 dialysed)
Enzyme 19AP (5% suspension dialysed and centrifuged)	12–15	4×10^3 dialysed
Helix aspersa (stomach contents after starvation)	370	6×10^3
Helix pomatia (stomach contents after starvation)	625	10^4

fulvum mycelium [0·43 g. of N/l., 1·225 g. of total carbohydrate (orcin)/l., total dry matter 6·8 g./l.] was analysed, using samples of 0·1, 0·2, 0·4, 0·6, 0·8 and 1 ml. The apparent acetylglucosamine contents/l. were 385, 345, 285, 177, 75 and 53 mg. The depression in colour formation with larger samples was so marked that a maximum colour developed with a sample volume of 0·4 ml., larger amounts giving less total colour. Neither mannitol nor starch at concentrations in a 1 ml. sample of 8 g./l. affected colour development. Tryptophan, tyrosine, alanine, glutamic acid, aspartic acid, β-phenylalanine and *p*-aminobenzoic acid all depressed colour formation at 2 g./l., and an appreciable effect was observed with glutamic acid, aspartic acid and alanine at 0·2 g./l. The effect described above with the *P. griseofulvum* extract could be reproduced with a solution containing 5 g. of tryptophan and 0·35 g. of acetylglucosamine per litre. The effect of glutamic acid in colour depression was about five times that of tryptophan. On paper chromatography the main amino acid component of the extract appeared to be glutamic acid.

Urea increases colour formation by acetylglucosamine, the position of the main absorption peak being little affected, though its height is increased. Increased absorption below 500 mμ. results in the final colour being more orange. Interference by urea can be detected not only by the change in hue, but, more objectively, by the quotient of absorption in the region of 465 and 540 mμ. (Ilford filters 622 and 625). For acetylglucosamine the quotient is about 0·3; with 0·01 M urea in the sample it rises to about 0·4, and reaches 0·85 with 0·5 M urea. Since some extracts of *L. giganteum* contained 0·3 M urea the effect is of some importance. It was found that urea in concentrations up to M had no effect on the course of hydrolysis of chitin by dialysed extracts of *L. giganteum*; consequently dialysed extracts were used wherever possible in order to avoid interference not only by urea but by other substances (such as amino acids) of low molecular weight. Dialysis of extracts at room temperature led to a slight (approx. 10 %) loss of activity.

Properties of Lycoperdon *chitinase*

Stability. The enzyme from *Lycoperdon* sp. is relatively stable in the crude or dialysed state. The hydrolysis of an excess of chitin by 0·03 ml. of a *L. pyriforme* extract was followed over a period of 88 days at 37°. At the end of this period hydrolysis was proceeding at one-quarter of the initial rate. That this decrease in rate of hydrolysis was probably due to inactivation of the enzyme rather than increased resistance of the insoluble substrate is indicated by determinations of loss of activity in the absence of substrate. *L. pyriforme* extract (0·5 ml.), after dilution to a total volume of 5 ml., 0·08 M in acetate at pH 5, was kept at 37° for 31 weeks. At the end of this period 1·33 % of the original activity was present; if a rate of inactivation proportional to the concentration of active enzyme is assumed, 25 % would have remained at the end of about 70 days and 17 % at the end of 88 days. Thus loss of activity is not markedly affected by the presence of substrate.

The half-life of about 40 days at 37° is increased to about 230 days when dialysed extract is stored at 5° in the presence of toluene. In the absence of toluene and of visible contamination the half-life was reduced to about 110 days. The effect of toluene and pentachlorophenol on stability and activity was determined, since Basu & Whitaker (1953) have found that toluene had an inhibitory effect on cellulase. Toluene and pentachlorophenol had no appreciable effect on activity when the period of enzyme action was 2 hr. or 4 days, provided that they were present in solution. The presence of sufficient toluene to form a separate phase was also without appreciable effect, but the presence of solid pentachlorophenol led to some inhibition in the 4-day experiments. Storage for 31 weeks at 5° resulted in a greater loss of activity when pentachlorophenol was the antiseptic than when toluene was used.

Heat inactivation. Dialysed *L. pyriforme* extract was heated by immersion in a water bath for 15 min. and then cooled in water to room temperature. Temperatures of 30°, 40°, 45°, 50°, 55°, 60° and 70° were used. The heated enzyme (0·5, 0·75; 1·0 and 1·25 ml.) and an unheated control, all diluted 1 in 5, were set up with *Sepia* chitin as described earlier. After 4 hr. the amount of acetylglucosamine liberated was determined. A plot of log (amount of acetylglucosamine formed) against the temperature to which the enzyme had been exposed gave a family of four approximately parallel straight lines. It was deduced from the plot that 50 % of the enzyme would be inactivated under the conditions used at 51°, 90 % at 56° and 99 % at 59°. Measurable inactivation with this short time of heating apparently begins soon after 40°.

The effect of protein. Whitaker (1952) showed that the hydrolysis of cellulose by *Myrothecium* cellulase proceeds faster in the presence of a number of proteins. His cellulase was a well-purified preparation containing little non-cellulase protein. Since the *Lycoperdon* extracts got by spinning off the extracellular fluid formed during ripening had low protein contents and high chitinase activity, the effect of added protein on the rate of hydrolysis of chitin was examined. It was found that added protein caused a sharp increase in the rate of hydrolysis of chitin by some preparations.

In comparing the activity of different preparations containing an enzyme it is usual to use a sub-

strate concentration high enough to ensure direct proportionality of the measured effect of enzyme action with the amount of enzyme used. Since the chitinase solutions used contained protein which appeared to have an activating effect, it was important to determine whether this effect was on the enzyme or on the substrate. If it were on the enzyme, then, with a fixed amount of substrate, it would be expected that proportionality would be achieved. If, however, the protein effect were directed at the substrate, increasing amounts of an impure enzyme solution might have more and more effect on the fixed amounts of substrate, and apparent enzyme content per unit volume might increase as increased volumes were used in testing. The latter effect was found to occur in some circumstances and the effect of added protein was therefore investigated more fully.

A dialysed *L. pyriforme* extract containing 100 mg. of protein N/l. was used at a dilution of 1 in 100. The protein solution was dialysed cow serum containing 7 g. of protein N/l.

Varied protein concentration. With 1 ml. of chitin suspension (1 g. of chitin N/l.) and 1 ml. of diluted enzyme (total protein N, 1 μg.) added cow serum produced a marked effect in the rate of chitin hydrolysis. The effect was measurable when 7 μg. of protein N were added (final concentration 1·4 mg./l.), the rate was doubled with 70 μg. and increased by a factor of 4·6 by 700 μg. The increases were followed over 5 days' incubation and the rates at each concentration remained approximately constant.

Varied substrate and protein concentration. Substrate concentrations of 0·1, 0·3, 0·6 and 1·0 ml. of chitin suspension/5 ml. were used. The increase of substrate concentration in the absence of added protein or the presence of protein at the 7 μg. level resulted in a slight increase in rate of hydrolysis (50 % over the full range). A similar increase occurred with 70 μg. of added protein N, though owing to the greater rate of hydrolysis it was quantitatively more marked. At the 700 μg. level of added protein the increase was greater (130 % over the full range of substrate concentration).

Varied enzyme and protein concentration. Serum concentrations were varied by adding 1, 3, 10, 30, 100 and 300 μg. of protein N. Enzyme concentrations were varied by adding 1·1, 3·3 and 10 μg. of protein N of the extract to each final volume of 5 ml. Substrate concentration was 100 μg. of chitin N/5 ml. After incubation for 24 hr. in the absence of added protein the increase in the amount of chitin hydrolysed by increasing amounts of enzyme was greater than would be expected. In the presence of 30 μg. of added protein N the hydrolysis effected was proportional to the amount of enzyme used. At higher concentrations hydrolysis per unit volume of enzyme fell off as more enzyme was used. Thus a family of curves was got by plotting enzyme concentration against chitin breakdown. At low protein concentrations the curves were concave and then with increasing protein concentration presumably passed through a straight-line form to give typical convex curves. Three typical curves are illustrated in Fig. 4.

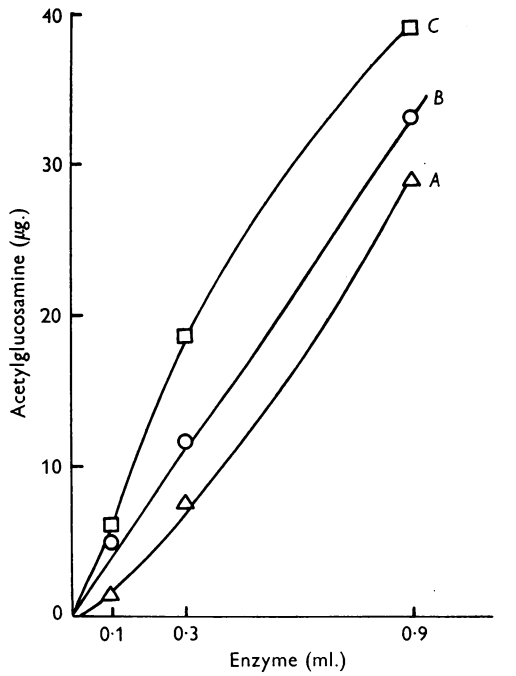

Fig. 4. Effect of added protein on hydrolysis of chitin by *L. pyriforme* chitinase. Time of incubation 24 hr.; 100 μg. of chitin N, 1·1, 3·3 and 10 μg. of total protein N of enzyme solution in 5 ml. *A*, no added protein; *B*, 30 μg. of added protein N; *C*, 300 μg. of added protein N.

Effect of sequestering agents. It was thought possible that the protein effect might in part be due to an immobilization by the protein of metal ions that might inhibit hydrolysis. The addition of histidine to the medium had an inhibitory effect on the rate of hydrolysis. The effect was marked at a final concentration of 200 mg./l. (rate was 65 % of control) and apparent at 20 mg./l. (90 % of control). Ethylenediaminetetraacetic acid had no effect on the rate of hydrolysis at the same concentrations. At pH 7 neither substance had any effect. Since ethylenediaminetetraacetic acid is more effective as a sequestrant at pH 7 than at pH 5, and in order to avoid assay at a pH at which enzyme activity is low, a mixture of dialysed enzyme, phosphate buffer pH 7 and ethylenediaminetetraacetic acid (10 mg./ml.) was dialysed against distilled water at 4°.

The final pH of the mixture containing ethylenediaminetetraacetic acid was 6·75, and of a control without the sequestering agent 7·05. After this treatment both solutions had lost about three-quarters of their activity when tested at pH 5, but there was no activating effect attributable to the ethylenediaminetetraacetic acid treatment. The protein-activation effect was a little more pronounced on the enzyme that had been treated with ethylenediaminetetraacetic acid. Citrate in final concentrations of 0·0002–0·01 M had no effect on the course of hydrolysis of chitin by the enzyme.

pH *optimum*. In the absence of added protein the sample of *L. pyriforme* chitinase used in these experiments had a pH optimum in the region of 5·0. The addition of bovine plasma albumin fraction V (The Armour Laboratories) in a final concentration of 2 mg./l. resulted in a shift of the optimum to pH 4·5 and an activation at all pH values between

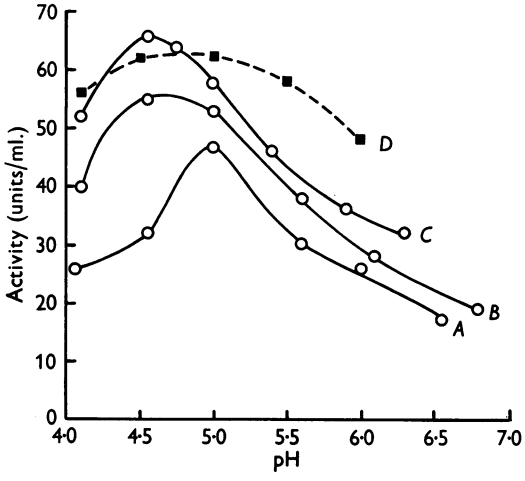

Fig. 5. Effect of protein on the rate of hydrolysis by, and the pH optimum of, a *L. pyriforme* extract. *A*, *B*, *C*, 11 μg. of protein N in extract; *D*, 110 μg. of protein N in extract. *A*, No added protein; *B*, 10 μg. of bovine plasma albumin (fraction V) added; *C*, 100 μg. of bovine plasma albumin (fraction V) added; *D*, no added protein.

4 and 7. Further activation was produced at a level of 20 mg./l., though the pH optimum remained in the region of 4·5. With ten times as much enzyme (110 μg. of total protein N) the pH optimum was at about pH 5 and there was no activation or shift in the position of the pH optimum by added albumin (Fig. 5). The activating effect of the protein varies considerably with the pH at which digestion occurs. An extract of *L. giganteum* (about 550 μg. of total protein used in tests) showed no protein effect and had an optimum pH at 4·5.

End products of enzyme action. The action of the enzyme on chitosan has been discussed above. The end product of the action of the chitinase extracts with chitin as substrate appears to be acetylglucosamine alone. No chitin-hydrolysing extracts from fungi, earthworms, eelworms, soil amoebae, or bacteria tested gave any evidence that glucosamine was among the end products, even though the active extract was allowed to work for months. Active preparations incubated at pH 5 with acetylglucosamine apparently leave it unchanged. A disaccharide does not seem to be a stable end product. The extracts from *Lycoperdon* sp. have been repeatedly used in the analysis of chitin-containing material and, provided that the material is suitably prepared, the yield of acetylglucosamine agrees well with that expected from chemical analysis (Tracey, 1955).

DISCUSSION

One of the main objects of the work described here was the discovery of a source of active chitinase for analytical purposes. It was obvious early that the fluid produced during the ripening of *Lycoperdon* sp. was very suitable. It is of high activity and can be stored for a year or more without losing enough activity to affect its use in practice. Hitherto *Helix* digestive juice has seemed the best source of the enzyme, but the *Lycoperdon* material can be got in vastly larger amounts with very little trouble and has the advantage that very little other material is associated with it.

Since no attempts at purification of the enzyme have been made, little can be said of its properties. The activity detected in the extracts may be the result of the action of one or more enzymes. It is possible that only one is involved, as with the breakdown of cellulose by some *Myrothecium* extracts (Whitaker, 1953; Kooiman, Roelofsen & Sweeris, 1953; but see Gilligan & Reese, 1954). Some evidence pointing to the presence of only one enzyme is provided by the observation that acetylglucosamine has a greater inhibitory effect than other sugars on the initial stages of chitosan breakdown. In similar experiments with fungal cellulases and the soluble cellulose derivative carboxymethylcellulose, Reese, Gilligan & Norkrans (1952) found that sugars other than cellobiose had little or no effect on the rate of loss of viscosity of the substrate. Enebo, Sandegren & Ljungdahl (1953) found that malt cellulase in unpurified extracts was unaffected by sugars other than cellobiose. Since in these viscometric assays glucose had little or no effect on viscosity changes, whereas the effect of cellobiose was pronounced, it was concluded that the end product of the enzyme(s) responsible for viscosity reduction was cellobiose. Since the effect of chito-

biose on viscosity reduction was not determined, no direct comparison of cellulase and chitinase is possible on these grounds; but it appears significant that with chitinase the monomer (acetylglucosamine) was inhibitory, whereas with cellulase it was not, though the dimer, cellobiose, was. It appears almost certain that the end product of the action of these unpurified extracts on chitin itself is acetylglucosamine. The yield of acetylglucosamine determined by a modification of the colorimetric method of Morgan & Elson (1934) was that expected from the nitrogen content of the chitin. It is known that no colour is developed by *NN'*-diacetylchitobiose, though when acetylglucosamine is substituted other than on carbon atom 4 it may be developed (Kuhn *et al.* 1954). Nevertheless, the fact that oligosaccharides containing acetylglucosamine might develop colour means that the figures for acetylglucosamine formed by prolonged action of chitinase on chitosan do not necessarily refer to free acetylglucosamine. There is consequently little point in speculating, on the basis of the figures quoted, about the type of linkage attacked by the enzyme and the exact reason for the reduced susceptibility of chitosan to hydrolysis.

There seems no doubt, however, that deacetylation of acetylglucosamine by the enzymes studied does not occur. Hackman (1954) found that the glucosamine present in the products of the action of *Helix* enzymes on chitin could be accounted for by the known degree of deacetylation of the substrate that occurred during its preparation, and Reynolds (1954) found no glucosamine present after enzymic breakdown of chitin by *Streptomyces* species. The end products of this latter action were *N*-acetylglucosamine and *NN'*-diacetylchitobiose.

The demonstration that added protein may cause marked changes in the rate of substrate breakdown and in the optimum pH for hydrolysis means that, as with cellulase, considerable care may be necessary before quantitative comparisons are made between chitinases from different sources, and it may even be a prerequisite for accurate work that the enzymes be first brought to a pure state. These effects are similar to those reported for *Myrothecium* cellulose by Whitaker (1952) and Basu & Whitaker (1953). They suggested that the protein effect was unlikely to be due to protection of the enzyme from denaturation or to an interaction with an inhibitor present in the enzyme solution or assay medium. The ineffectiveness of chelating agents in modifying the effect of protein on chitinase preparations and the stability of the enzyme in the assay medium used suggest that the explanations rejected by Whitaker are unlikely to hold in the instance of chitinase. Basu & Whitaker (1953) suggest that the protein effect and the effect of a number of dyes on the action of the *Myrothecium* enzyme may be explained by a change in surface potential of the insoluble substrate that results from adsorption of compounds from solution.

An unequivocal proof of the suggestion that the primary effect of the protein is on the substrate may be difficult to get. The event of a simultaneous collision of substrate, activator and enzyme need not be considered, and it is therefore a question of deciding whether an interaction between enzyme and activator, between substrate and activator or between enzyme–substrate complex and activator is the effective step in increasing the rate of substrate breakdown in the system. The demonstration of activation by a substance completely adsorbed on the substate would throw doubt on the first and third possibilities. Unfortunately none of the basic dyes found to stimulate *Myrothecium* cellulase were completely adsorbed on the substrate and nor was serum albumin. Nevertheless, the results of Whitaker (1952), of Basu & Whitaker (1953) and those described earlier suggest that the substrate–activator interaction may be the important step in the stimulation of cellulase action. If definite proof of this suggestion were obtained, it would perhaps be unwise to risk confusion by referring to an 'activating' effect of protein in such systems, as this has come to mean an effect on the enzyme itself, either direct or by removing inhibitory substances that would otherwise combine with it. It is suggested that effects of the nature discussed here may be distinguished by the term 'opsonic', with the direct implication that the effect is directed to the substrate rather than to the enzyme. The root 'opson' (from *opsono* 'I render palatable') was introduced by Almroth Wright in describing the 'activation' of leucocytosis by substances in the blood that affect the foreign bodies ('substrates') rather than the active agents the leucocytes ('enzymes').

SUMMARY

1. Fungi of the *Lycoperdon* group have been found a useful source of active chitinase.

2. Active chitinase solutions may show a response to added protein similar to that found with fungal cellulase.

3. Methods for the viscometric and hydrolytic assay of chitinase are described.

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The Determination of Glucosamine and Galactosamine

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An analytical procedure for the quantitative determination of glucosamine and galactosamine was described by Elson & Morgan (1933). The method was based on the observation by Pauly & Ludwig (1922) that in alkaline solution at 100° the amino sugars react with acetylacetone to form a chromogenic material which gives a chromophore or chromophores on treatment in acid solution with ethanolic *p*-dimethylaminobenzaldehyde. The development and use of photoelectric spectrophotometers during the intervening years has increased the sensitivity and accuracy of technique very considerably, but although many modifications of procedure designed to improve the method have been described there has, in fact, been no fundamental change in the procedure originally employed. The method given in this paper has been used for many years and has given satisfactory results. The procedure is now described because we have frequently referred, in publications from this Laboratory, to a modification, whose details have not been published, of the technique described in the original paper. To ensure that a reliable determination of amino sugar is achieved, it is imperative that an established and rigid technique is used, since slight variations in procedure, especially those affecting the pH during the condensation with acetylacetone, lead to marked differences in the colour intensity finally obtained.

In the extensive literature on this reaction papers by Sørensen (1938), Blix (1948), Schloss (1951) and Belcher, Nutten & Sambrook (1954) describe work done on the estimation of amino sugar itself, and an account of the determination of the amino sugar content of a variety of naturally occurring substances is given by Boyer & Fürth (1935), Jorpes (1935), Palmer & Meyer (1935), Dakin & West (1935), Dakin, Ungley & West (1936), Nilsson (1936), Lüstig & Ernest (1937), Palmer, Smyth & Meyer (1937), Masamune & Nagazumi (1937), Dakin, Young & Krauss (1938), West & Clark (1938), Hewitt (1938), Hamasato & Akakura (1941), Hadidian & Pirie (1948), Ogston & Stanier (1950), Johnston, Ogston & Stanier (1951), Cessi (1952) and Anastassiadis & Common (1953). Reference is made later to work done on other aspects of the determination (see Discussion).

EXPERIMENTAL

Materials and methods

Acetylacetone reagent. Redistilled acetylacetone (b.p. 138–140°, 1 ml.) is dissolved in 50 ml. of 0·5 N-Na_2CO_3 solution. The reagent is prepared immediately before each estimation and is stable for 2–3 hr. at 18°.

Ethanol. This is distilled after drying for 24 hr. over freshly heated calcium oxide.

p-*Dimethylaminobenzaldehyde reagent* (Ehrlich's reagent). Commercial-grade *p*-dimethylaminobenzaldehyde is purified as described by Adams & Coleman (1948) and finally reprecipitated from ethanol by the addition of 50–75% of water. A suitable material is colourless to pale yellow and melts at 73°. The aldehyde (0·8 g.) is dissolved in 30 ml. of ethanol and 30 ml. of conc. HCl (A.R., sp.gr. 1·18) are added. The solution should be free from insoluble matter and pale yellow; it keeps well when stored at −10°.