STUDIES ON A NEW CELLULASE PREPARATION FROM PENICILLIUM

II. Properties and Action upon Different Substrates

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Received July 2, 1959

The properties of a new cellulase preparation from *Penicillium* were studied. In particular, its action upon some vegetable substrates was investigated under varying experimental conditions, and the possibility of its containing at least two enzymes is discussed. Comparative studies were made with an enzyme preparation from *Aspergillus oryzae*.

Oral administration of digestive enzymes has been used in the treatment of pancreatic insufficiency and fermentative dyspepsia. Most therapeutic preparations contain trypsin, lipase and amylase from pancreas, but some contain mold enzymes, primarily amylase and cellulase.

According to Grassmann and Rubenbauer[1] cellulase breaks down vegetable cell walls and thus facilitates digestion. They used enzymes isolated from *Aspergillus oryzae*, and measured their cellulolytic activity against the insoluble substrates hydrocellulose and lichenin. Others have employed soluble cellulose derivatives as substrates.

The soluble cellulose derivatives are hydrolysed more rapidly by cellulases than is cellulose under similar experimental conditions. This is due, in the view of Karrer[2] and Walseth[3], to the crystalline state of the substrate; though the phenomenon has also led to the postulation of a multi-enzyme mechanism in the decomposition of cellulose. According to Reese and others[4,5] a cellulase preparation from *A. oryzae* contains both an enzyme that alters the physical state of cellulose fibres and liberates polymer chains, and one which catalyses the hydrolysis of the latter to reducing sugar. The last-named enzyme is assumed to break down carboxymethylcellulose also.

Other investigations lend support to the view that several enzymes are responsible for the effect of cellulase preparations. Jermyn[6] showed, following paper chromatographic separation, that impure *A. oryzae* preparations have no fewer than eight components which act upon carboxymethylcellulose. Reese and Gilligan[7] found, with a chromatographic technique, three components in *Myrothecium* filtrate, while Miller and Blum[8] observed in the same product at least eight components with a cellulolytic action.

Heiwinkel, Lindvall and Reizenstein[9] reported that heterogenous cellulases are not identical in their actions. Vegetables treated with identical amounts of cellulase, as measured by a viscometric method[10], were digested both more rapidly and more extensively by the *Penicillium* preparation than by a commercial *Aspergillus* preparation.

Vegetable decomposition produced by a cellulase preparation from *A. oryzae* was studied in detail by Silberschmidt[11]. He found macroscopically that the enzyme digested cucumber, kohl-rabi, common radish, and turnip radish. Microchemical methods revealed that the sensitivity of vegetable substrates to cellulose-colouring substances was lost earlier than that to pectin-colouring subtances. These changes proceeded parallel with the lytic action on the cell walls. Decomposition of vegetables was studied also by Holden[12] using tobacco leaves as substrate and various types of enzyme systems.

The investigation herein reported is concerned with a new cellulase preparation. Special attention has been given to its action on carboxymethylcellulose and on various vegetables. The preparation is identical with that used by Heiwinkel and others[9] in their studies on gastrointestinal cellulolytic activity after oral administration in man, and was produced microbiologically from a special strain of *Penicillium notatum*. It was purified to an activity of 5 units per milligram, measured by the viscometric method of Eriksson and Lindvall.[10]

The actions of the *Penicillium* cellulase were also compared in some respects with those of various preparations from *Aspergillus*. The designations and nature of the preparations used are listed in Table I.

TABLE I

Preparation	Formula	pH optimum	Cellulase units per tablet
A	Conc. e cult. penicill. spec.	4·15	200
B	Conc. e cult. penicill. spec. Pancreatin Diastase	4·15	135
C	Extr. aspergill. oryzae	3·85	155
D	Extr. aspergill. oryzae Pancreatin.	3·85	75
E	Extr. mycel. aspergill. Papainum pur. Cystein. hydrochl.	3·85	45
F	Extr. mycel aspergill. spec. diff. pur. sicc. Extr. gl. pancreat. pur. sicc. Papainum pur. Cystein. hydrochl. Faex sicc.	3·85	30
G	Aspergill. oryzae sicc. dep. Extr. pancreat. Extr. fellis bovis	3·85	12

Experiments and Results

pH Optimum for Enzymatic Activity

The pH optimums for enzymes from *Aspergillus* and *Penicillium* in the decomposition of carboxymethylcellulose were determined viscometrically with the use of McIlvaine's buffer or 0·05 mole acetate buffer at different pH. Cellulase from *Penicillium* has its optimal activity at pH 4·15, and enzymes from various commercial *Aspergillus* preparations (according to the makers' formula, *A. oryzae*) at a somewhat lower pH of 3·85.

The pH optimums for the different enzymes are sharply defined, although there is relatively high activity even at pH 5 to 6, especially with the *Penicillium* preparation.

The cellulolytic activity of the medicinal forms (tablets) of the various preparations was measured at the respective pH optimums, using the viscometric method devised by Eriksson and Lindvall[10]. The results are in Table I. Some of the *Aspergillus* preparations showed fluctuating values, but only the highest in each instance is tabulated.

pH Optimum for Thermal Stability

Solutions of the enzyme preparations were made by extraction with water. After adjustment to different pH values by addition of known amounts of hydrochloric acid or sodium hydroxide, the specimens were exposed at 37° for 30 minutes. After neutralisation, the cellulase activity was determined at the pH optimums, that is 4·15 for *Penicillium* cellulase and 3·85 for *Aspergillus* cellulase. The results are presented in Figure 1, which shows optimal stability at pH 5 to 7 for the *Penicillium*, and at pH 4·0 to 6·5 for the *Aspergillus* preparation.

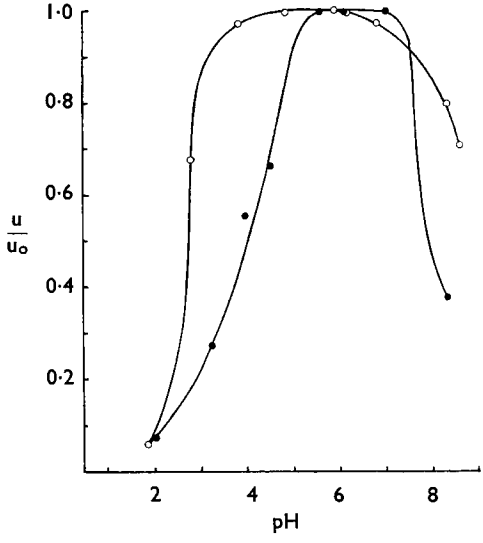

FIG. 1. Persisting activity of the *Penicillium* (preparation A) and *Aspergillus* (preparation C) enzymes after 30 minutes' treatment at 37° and at different pH.

○——○ Aspergillus enzyme.
●——● Penicillium enzyme.

Temperature Optimum for Enzymatic Activity

The enzymatic activity at temperatures between 30 and 65° was determined with carboxymethylcellulose as the substrate. The reaction time was 5 minutes at these temperatures. For determinations at 32 and 40°, both substrate and enzyme solution were heated to the requisite degree

before being combined. For determinations at higher temperatures the substrate had been heated so as to produce the required temperature when combined with enzyme solution of room temperature.

Enzymatic digestion of the carboxymethylcellulose was subsequently terminated by adding mercury acetate in an amount to give a concentration of 0·001 mole, which inactivates the enzyme completely (*cf.* Grassmann and others)[13]. The viscosity of the reaction mixture was then measured at 37°, and the activity calculated by the method of Eriksson and Lindvall[10]. The results are in Figure 2B, from which it will be seen that the enzyme had its optimal activity at 50 to 55°.

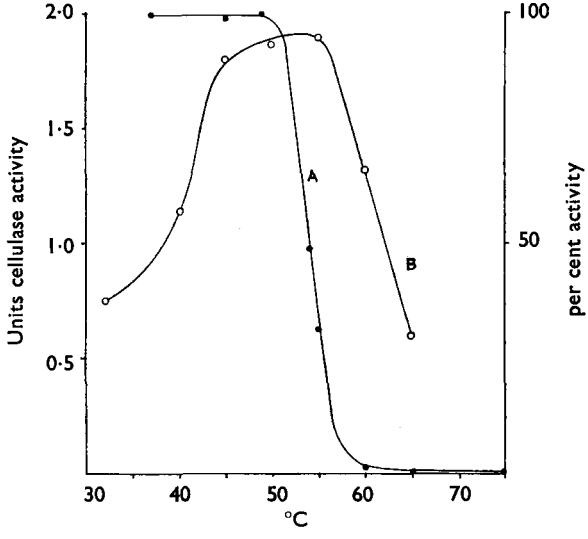

FIG. 2A. Persisting activity of the *Penicillium* enzyme after 30 minutes' heating to different temperatures. B. Enzymatic action of the *Penicillium* preparation upon carboxymethylcellulose at different temperatures.

Thermal Inactivation of the Enzyme

An aqueous solution of the *Penicillium* enzyme was treated in a thermostat at various temperatures for 30 minutes. The solution was then rapidly cooled to 37° and the activity determined viscometrically. The results, which are presented in Figure 2A, show that under these conditions the enzyme is totally inactivated at temperatures exceeding 60°.

The rate of deactivation was measured by heating solutions of the *Penicillium* enzyme to 50, 55, 60 and 65° for varying periods up to 30 minutes. After rapid cooling, the persisting activity was determined in the usual manner. Figure 3A shows the results. The values after 5 minutes' deactivation must be considered unreliable, since temperature equilibrium does not occur instantaneously.

Relatively high enzymatic activity could also be shown to be present at pH 5 to 6, especially with the *Penicillium* enzyme. This suggests the

presence of two enzymes with different pH optima. To find out if the thermal sensitivity corroborated such an assumption, a solution of the enzyme was exposed to temperatures of 55 and 60° for varying periods, after which its activity was determined at different pH values. The results (Fig. 3B) do indeed point to the existence of two cellulolytic enzymes of differing thermal stability, one of which (optimum at pH 4·15) appears to be more sensitive to heat than the other, which has its optimum at approximately pH 6.

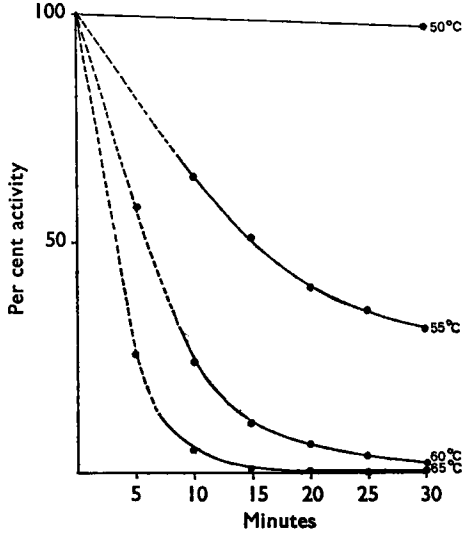

FIG 3A. Persisting activity of the *Penicillium* enzyme after thermal treatment for varying periods at 50, 55, 60 and 65°.

Action of the Enzyme on Different Substrates

Heiwinkel and others[9] found, in regard to digestion of vegetables, a substantial difference per viscometric cellulase unit between the cellulase used in this study and preparations from *Aspergillus*. Their experiments were made at pH 4·2.

With the aim of establishing whether the decomposition of vegetables was dependent on pH, potato cubes measuring 5 mm. per side were digested by 0·05, 0·5 and 5 viscometric enzyme units per ml. from *Penicillium* and *Aspergillus* respectively. Twenty-five g. of potato cubes were shaken in 100 ml. McIlvaine's buffer at pH ranging from 3·0 to 7·5, diluted with an equal volume of aqueous solution of cellulase. The duration of treatment was 2 hours and the temperature 37°. Undigested cubes were filtered off with a Büchner funnel of Jena Duran glass having a filter 5·5 cm. in diameter, with 0·5 by 3 mm. perforations. After filtration, the fragments collected on the filter were weighed. The results are shown in Figure 4, from which it is evident that digestion of potato was

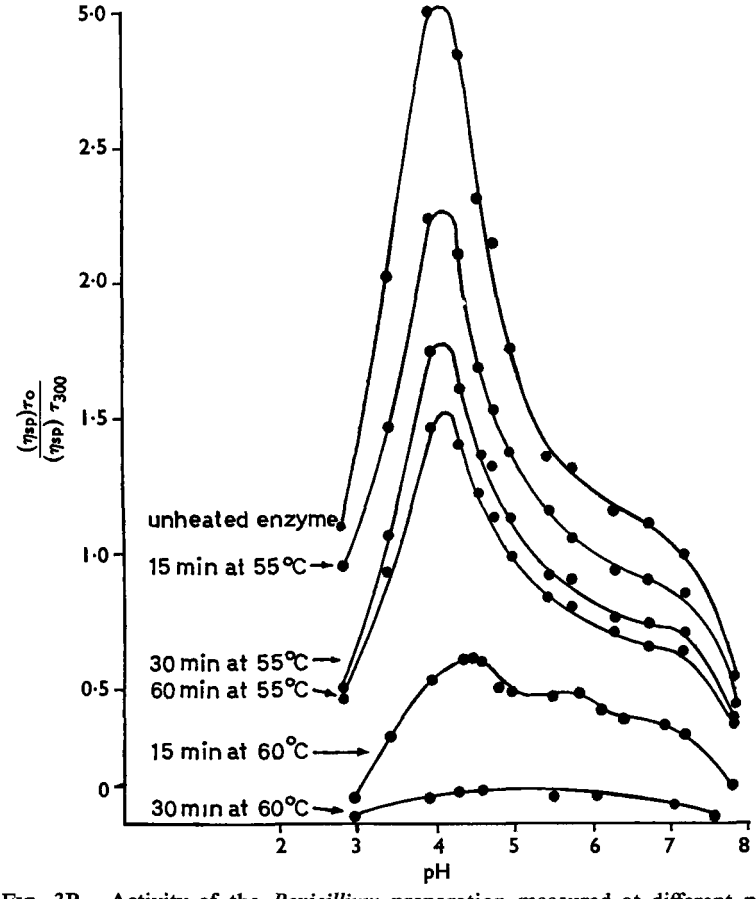

FIG. 3B. Activity of the *Penicillium* preparation measured at different pH after heating for varying periods.

less with *Aspergillus* than with *Penicillium* enzyme, not only at pH 4·2, but throughout the pH range tested.

The *Penicillium* preparation, as mentioned in the foregoing, seems to contain more than one enzyme. The above experiments with potato lend weight to this supposition. To secure further evidence a number of other vegetables were digested at pH values in the range of 3 to 8. Twenty-five to 30 g. of finely chopped carrot, white cabbage, apple and cauliflower were each treated in the same way as potato. The digestion was determined after 3 hours' treatment with 25 enzyme units per ml. It appears evident from Figure 5 that the preparation contains at least two enzymes, one with its optimum at approximately pH 6 and the other at approximately 4·5. In conjunction with the experiments on white cabbage, digestion of that substrate by the *Aspergillus* enzyme was subjected to comparative study. Here too, a substantial difference was noted between enzyme preparations from *Aspergillus* and from *Penicillium*.

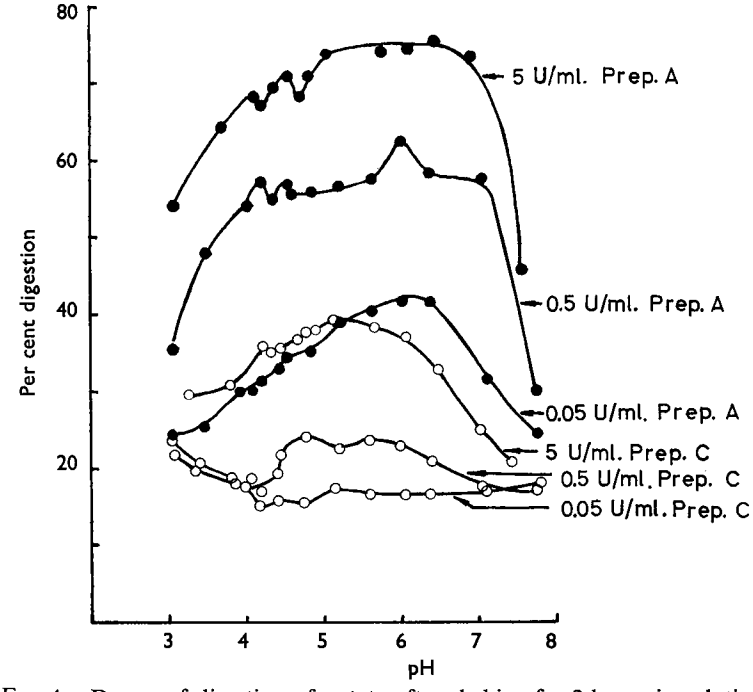

FIG. 4. Degree of digestion of potato after shaking for 2 hours in solutions of varying enzyme concentrations from *Penicillium* (preparation A) and *Aspergillus* (preparation C) at different pH and at 37°.

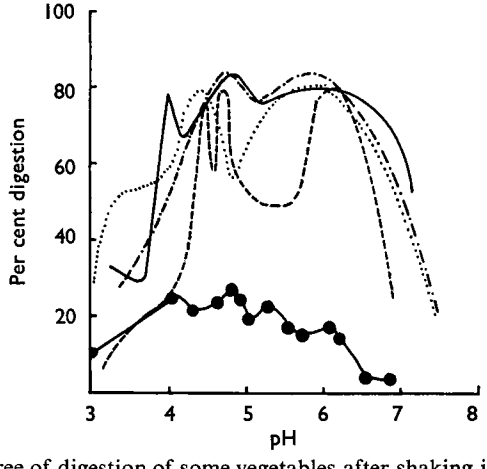

FIG. 5. Degree of digestion of some vegetables after shaking in solution containing 25 units of the *Penicillium* enzyme per ml. for 3 hours at 37°; also, for cauliflower, in a solution of the *Aspergillus* enzyme (preparation C) under identical experimental conditions.

——— Apple. — — Cauliflower.
—·— Carrot. . . . White cabbage.
●—● Prep. C.

Discussion

According to our results the cellulase preparation from *Penicillium* contains at least two enzymes with the ability to digest vegetables. These enzymes have not only disparate pH optima, but also differ in their thermal sensitivity.

As regards the action upon vegetables, the pH optimum diverges from that obtained with carboxymethylcellulose as substrate. The question whether this difference in pH activity is due to the presence of several enzymes which are substrate-specific, or to the physical state of the different substrates like solubility, charge, or crystallinity, will be the subject of a future investigation. The cellulase prepration from *Penicillium* differs in many respects from the *A. oryzae* preparation. With carboxymethylcellulose as substrate, the two have somewhat disparate pH optimums; moreover, the *Penicillium* cellulase shows a better action at pH 5 to 6 than the *Aspergillus* preparations that we studied. There are certain discrepancies also in pH stability. The principal difference, however, is in the digestion of vegetables, in which respect the *Penicillium* cellulase is considerably more active. When tested on potato, for instance, it has, in a concentration of 0·05 viscometric units per ml. approximately the same activity as the *Aspergillus* cellulase in a concentration of 5 viscometric units per ml. Similar findings were recorded by Heiwinkel and others[9], using other vegetables. The cause of these discrepancies between the *Penicillium* and *Aspergillus* preparations is not clear, but probably it is to be sought in differing enzyme systems in the preparations. The purity is possibly of some significance.

References

1. Grassmann and Rubenbauer, *Münch. Med. Wschr.*, 1931, **78**, 1817.
2. Karrer, *Kolloid-Z.*, 1930, **52**, 304.
3. Walseth, Thesis, Institute of Paper Chemistry, Appleton, Wisconsin 1948.
4. Reese, Siu and Levinson, *J. Bact.*, 1950, **59**, 485.
5. Reese and Levinson, *Physiologia Plantarum*, 1952, **5**, 345.
6. Jermyn, *Australian J. Sci. Research*, 1952, *Ser. B*, 5, 433.
7. Reese and Gilligan, *Arch. Biochem. Biophys.*, 1953, **45**, 74.
8. Miller and Blum, *J. biol. Chem.*, 1956, **218**, 131.
9. Heiwinkel, Lindvall and Reizenstein, *Gastroenterol.*, in press.
10. Eriksson and Lindvall, *J. Pharm. Pharmacol.*, 1959, **11**, 00.
11. Silberschmidt, *Münch. Med. Wschr.*, 1931, **78**, 1819.
12. Holden, *Biochem. J.*, 1950, **47**, 399.
13. Grassmann, Zechmeister, Tòth and Stadler, *Ann. Chemie*, 1933, **503**, 167.