obtained with an inoculum size which does not give maximum mycelial dry weight. The pectic enzymes show a marked increase during the third day of growth, and this point is marked also by the virtually complete utilization of the sucrose originally present in the culture and by a discontinuity in the curve of mycelial dry weight.

SUMMARY

1. A method is described for the preparation of a mixture of pectic enzymes with cultures of *Aspergillus niger*.

2. The cultures were produced in stirred fermenters with a medium consisting of 0·05% of Na_2SO_4, 0·2% of NH_4NO_3, 2% of sucrose and 2% of pectin in a boiled extract of groundnut flour. Fermentation was continued for 5–6 days at 30°. The pH of the culture remained between 3 and 4.

3. The preparations induced virtually complete hydrolysis of pectin.

4. Enzymic preparations obtained from the culture fluid by precipitation with ethanol were more active, on a weight for weight basis, than commercially available materials.

We wish to acknowledge the award of research fellowships by the Istituto Superiore di Sanità. We are grateful to the Direction and staff of the pilot plant of the Institute for the provision of facilities and technical assistance. We would especially like to recognize the encouraging guidance and help of Professor E. B. Chain, F.R.S.

REFERENCES

Ayres, A., Dingle, J., Phipps, A., Reid, W. W. & Solomons, G. L. (1952). *Nature, Lond.*, **170**, 834.
Brooks, J. & Reid, W. W. (1955). *Chem. & Ind.* p. 325.
Chain, E. B., Paladino, S., Ugolini, F. & Van der Sluis, J. (1954). *R.C. Ist. sup. Sanit.* (Engl. ed.), **17**, 61.
Clutterbuck, P. W., Lovell, R. & Raistrick, H. (1932). *Biochem. J.* **26**, 1907.
Czapek, F. (1902). *Beitr. chem. Physiol. Path.* **2**, 557.
Demain, A. L. & Phaff, H. J. (1957). *Wallerstein Labs Commun.* **20**, 119.
Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 138. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Jansen, E. F. & MacDonnell, L. R. (1945). *Arch. Biochem.* **8**, 97.
Jarvis, F. G. & Johnson, M. J. (1947). *J. Amer. chem. Soc.* **69**, 3010.
Kontio, P. (1950). In *Chem. Abstr.* **46**, 10274b.
Lullar, B. S. & Johar, D. S. (1953). *Curr. Sci.* **22**, 79.
McCready, R. M., Swenson, H. A. & Maclay, W. D. (1946). *Industr. Engng Chem. (Anal.)*, **18**, 290.
Mill, P. J. & Tuttobello, R. (1961). *Biochem. J.* **79**, 57.
Ozawa, J. & Okamoto, K. (1954). In *Chem. Abstr.* **48**, 7119f.
Paladino, S. (1954). *R.C. Ist. sup. Sanit.* (Engl. ed.), **17**, 145.
Patel, D. S. & Phaff, H. J. (1959). *J. biol. Chem.* **234**, 237.
Roe, J. H. (1934). *J. biol. Chem.* **107**, 15.
Saito, H. (1955). *J. gen. appl. Microbiol.* **1**, 38.
Saito, H., Yasuji, M. & Marumo, H. (1954). *J. agric. chem. Soc. Japan.* **28**, 810.
Shu, P. & Johnson, M. J. (1948). *Industr. Engng Chem.* **40**, 1202.
Somogyi, M. (1952). *J. biol. Chem.* **195**, 19.
Swangard, W. M. (1947). *F.I.A.T. Final Report* no. 910. Cited in Kertesz, Z. I. (1951). *The Pectic Substances*, p. 378. New York: Interscience Publishers Inc.
Takehana, H. & Ogura, N. (1955). *J. agric. chem. Soc. Japan.* **29**, 83.
Tanner, F. W. (1919). *Bacteriology and Mycology of Foods*, p. 50. New York: John Wiley and Sons Inc.
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
Whiffen, A. J. & Savage, G. M. (1947). *J. Bact.* **53**, 231.

Biochem. J. (1961) **79**, 57

The Pectic Enzymes of *Aspergillus niger*

2. ENDOPOLYGALACTURONASE

BY P. J. MILL AND R. TUTTOBELLO
International Centre for Chemical Microbiology, Istituto Superiore di Sanità, Rome, Italy

(*Received* 29 *September* 1960)

Endopolygalacturonase, as defined by Demain & Phaff (1957), is an enzyme hydrolysing the galacturonosidic links of pectic substances. It acts preferentially on pectic acid rather that on pectin in contrast with endopolymethylgalacturonase; the mechanism of attack is random and does not appear to go to completion, in contrast with exopolygalacturonase with its terminal mechanism of attack. Saito (1955) attempted to purify this enzyme from *Aspergillus niger* under the name of depolymeric galacturonase, but he nowhere expresses the activities of his preparations in terms of their protein content and as no criteria are given for the purity of the final preparation it is not

possible to say to what extent the purification was successful.

McCready & Seegmiller (1954) purified an endopolygalacturonase from a commercial enzyme preparation, Pectinol, whose source is unknown but presumably fungal. These authors tested the ability of their preparation to hydrolyse purified oligogalacturonic acids. The preparation acted against all those oligogalacturonic acids tested, including the dimer and trimer, but here the action was feeble. It was concluded that this endopolygalacturonase would hydrolyse any $(1 \rightarrow 4)$-α-link between two galacturonic residues with free carboxyl groups.

These results are largely in accordance with those obtained by Patel & Phaff (1959) with a well defined endopolygalacturonase obtained from the culture fluid of *Saccharomyces fragilis*, except that this enzyme did not attack digalacturonic acid.

In a definitive study of this enzyme from a particular mould it is therefore necessary to purify the material as extensively as possible, so as to exclude the catalysis of reactions by traces of exopolygalacturonase contaminating the preparation of endopolygalacturonase. This paper describes the partial purification of an endopolygalacturonase from culture fluids of *A. niger* in good yield and with a purification of more than 400-fold, and outlines some of the properties of this preparation, which does not hydrolyse digalacturonic acid.

EXPERIMENTAL

Methods and materials

Fermentations. The techniques employed for the fermentative production of a mixture of pectic enzymes are described in the preceding paper (Tuttobello & Mill, 1961). *A. niger* strain CH, which had been isolated from decomposing fruit, was used throughout.

Ethanol precipitations. These were carried out in vessels of 20 and 50 l. capacity provided with jackets, through which a refrigerant mixture was circulated. Ethanol (95%), precooled to −20°, was added slowly to the mixture from large tap-funnels. Efficient stirring was maintained throughout and the temperature was held as close to the freezing point of the mixture as possible, without the actual formation of ice, down to a minimum of −10°. Centrifuging was performed with a Sharples air-driven centrifuge fitted with a cooling coil.

Pectin and pectic acid. Apple pectin (Fluka A. G., Buchs, S. G., Switzerland) was used after being washed with 80% ethanol containing 0·05 N-HCl; it had a uronic anhydride content of 81% (by the method of McCready, Swenson & Maclay, 1946) and 7·6% of methoxyl (by the method of Myers & Baker, 1934).

Pectic acid was produced from this by alkaline saponification (Kertesz, 1951) and was precipitated three times with 60% ethanol. The precipitated gel was redissolved in water with the cautious addition of N-NaOH to produce a pH of 4·0. After centrifuging, the solution was freeze-dried and the product stored in a desiccator. The dry product contained 83% of uronic anhydride. A portion (2 g.) of this product was dissolved in water containing 0·5% of disodium ethylenediaminetetra-acetate, the pH was adjusted if necessary, and then the solution was diluted to a volume of 100 ml. This constituted the solution designated as '2% sodium pectate'. It was dispensed in test tubes in 5 ml. quantities and held at −20° until required; any of the thawed solution not used in the same day was discarded. A 1% solution of the sodium pectate at pH 4·8 and 30° had a relative viscosity of 6·0–6·5.

Separation of oligogalacturonides. Mixtures of oligogalacturonides were produced by the digestion of pectin with fungal-pectinase preparations: the progress of the reaction was followed chromatographically and it was stopped by heating when a suitable composition had been reached. The bulk of the galacturonides, other than the monomer, were precipitated as their strontium salts (Luh & Phaff, 1954). The free acids, generated by treatment with Amberlite IR-120 (H^+) cation-exchange resin, were separated on a column of De-Acidite FF resin (formate form) (Ashby, Brooks & Reid, 1955).

Assays of enzymic activity. The specific assay of endopolygalacturonase in the presence of other pectolytic enzymes is difficult; a viscometric method was employed, which, although affected by exopolygalacturonase, may be expected to be much more sensitive to endopolygalacturonase (Demain & Phaff, 1957). The tendency in recent years has been to evaluate viscosity assays by determining the percentage fall in viscosity of a mixture of enzyme and pectic acid after a fixed reaction period. The percentage fall in viscosity (A) has been defined by Roboz, Barrett & Tatum (1952) as:

$$A = \frac{V_0 - V_t}{V_0 - V_s} \times 100$$

where V_0 is flow time (sec.) of pectic acid + heat-inactivated enzyme; V_t, flow time (sec.) of pectic acid + enzyme; V_s, flow time (sec.) of inactivated enzyme + solvent. Logarithmic dose–response relationships were found with assays performed in this manner (Reid, 1952; Roboz *et al.* 1952; Saito, 1955). The use of this type of assay introduced two ambiguities: first, the relationship of viscosity to average molecular size is complex; and, secondly, endopolygalacturonase has markedly different rates of attack on substrates of different sizes, so that it is not surprising that a complex relationship connects the percentage fall in viscosity with the amount of enzyme present.

These difficulties may, however, be readily avoided by determining the period taken to perform a constant amount of hydrolysis. Both Fig. 1, and the results of Roboz *et al.* (1952) and Saito (1955), show that the time required to reach a value for A of 50% is inversely proportional to the concentration of enzyme over a considerable range of activities. Throughout this work one viscosity-diminishing unit of endopolygalacturonase activity has been taken as that amount of enzyme which reduces the viscosity of 1 ml. of a 1% solution of sodium pectate by 50% in 10 min. at pH 4·8 and 30°, i.e. the number of units present in 1 ml. of the reaction mixture under the stated conditions is $10/T_{50}$, where T_{50} is the time in minutes required to reach a value for A of 50%.

It was found that 0·1 ml. graduated pipettes could be used satisfactorily as viscometers. The stem of these was

bent through an angle of about 80° at about 5 cm. from the tip, and the pipette was clamped with this 5 cm. portion hanging vertically downwards. A mouthpiece attached to the other end of the pipette with rubber tubing allowed it to be filled. In use the enzyme was diluted in 0·2 M-acetate buffer, pH 4·8, and 0·5 ml. was placed in a 3 cm. × 0·5 cm. test tube; 0·5 ml. of 2% sodium pectate was placed in a similar tube. The tubes were immersed in a water bath maintained at 30° for 2 min., and then their contents were mixed, by pouring repeatedly from one tube to the other, and the time was noted. The mixture was drawn up into the pipette at intervals, and the period taken for the upper meniscus to travel between two selected points on the scale of the pipette was noted, together with the time at which the meniscus passed the mid-point of the scale. The flow time was also determined with buffer alone and with buffer and sodium pectate. Since the enzyme preparations were usually diluted 10^4- to 10^6-fold for assay, it was not usually necessary to use heat-inactivated enzymes. A value for A was calculated for each reading obtained and a plot of this against reaction time allowed T_{50} to be determined. Before and between readings the pipette was washed out five times with 0·1 M-acetate buffer, pH 4·8, held at 30°.

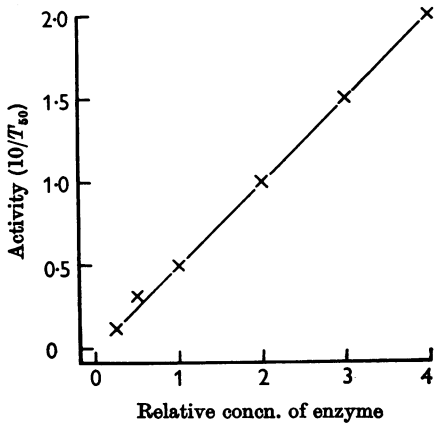

Fig. 1. Activity of various arbitrary concentrations of endopolygalacturonase measured by the time (T_{50}) required to induce a 50% fall in the viscosity of a 1% solution of pectic acid at pH 4·8 and 30°. The activities are expressed as $10/T_{50}$, with T_{50} measured in min.

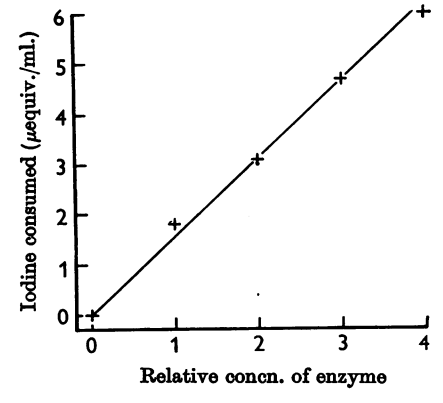

Fig. 2. Digestion of trigalacturonic acid by various arbitrary concentrations of enzyme determined by the colorimetric hypoiodite method on 0·1 ml. of a reaction mixture, containing originally 0·5% of substrate at pH 3·5, after digestion for 1 hr. at 30°.

The iodimetric method of Owens *et al.* (1952) was used in a slightly modified form to follow the release of reducing groups during pectolysis. The digestion was, as a matter of convenience, performed at 30° rather than 25° in view of the high ambient temperatures encountered in these laboratories. One unit of activity (PGu) is that which releases 1 m-mole of reducing sugar/min. from a 0·5% solution of sodium pectate at pH 4·0. Results, given as the percentage total hydrolysis, are based on the galacturonic anhydride content of the sodium pectate.

A simple colorimetric modification of the hypoiodite assay was evolved for the measurement of the release of reducing groups from purified oligogalacturonides; the method was economical of these substrates whose preparation is time-consuming. The entire reaction and assay were performed in 0·4 cm. × 10 cm. test tubes, which were selected as optically matched for use in an EEL colorimeter provided with a suitable adaptor. Replicate tubes were set up containing 0·5 ml. of a 1% neutralized solution of the substrate in 0·1 M-phthalate buffer, pH 3·5. These tubes, and suitable dilutions of the enzyme in the same buffer, were heated in a water bath at 30°, and then 0·5 ml. of enzyme was added to each tube. After a suitable period the reaction was stopped by the addition of 0·1 ml. of 4 N-Na_2CO_3; initial values were obtained by adding the Na_2CO_3 before the enzyme. After 0·5 ml. of 0·06 N-I_2 was added, each tube was stoppered. After a 20 min. reaction period, 3·4 ml. of 0·2 N-H_2SO_4 was added and the concentration of the residual I_2 was estimated in the colorimeter with filter no. 625. The colorimeter was calibrated with dilutions of standardized I_2 solution, and the assay was calibrated with galacturonic acid. The stoicheiometry of the hypoiodite assay was the same as with the normal technique (i.e. 1 m-equiv. of $I_2 \equiv 0{\cdot}51$ m-mole of sugar). Fig. 2 demonstrates the approximate linearity of the response obtained for the digestion of trigalacturonic acid by various amounts of enzyme.

Ultraviolet-absorption measurements. The extinctions of the enzyme preparations were measured at 280 mμ in a Uvispek spectrophotometer with a 1 cm. light-path. Protein concentration was taken to be proportional to extinction with $E^{1\%}_{1\,cm.} = 10$ (Dixon & Webb, 1958).

Chromatography. Chromatography of oligouronides was performed on Whatman no. 541 paper with, as solvent, the epiphase of butyric acid–acetic acid–water (200:1:200). Chromatograms were normally run for 24 hr. at 25° and the dried papers were developed by the $AgNO_3$-dip technique of Trevelyan, Procter & Harrison (1950). The series of spots produced by the chromatography of a partially hydrolysed sample of pectic acid gave a straight line when the logarithms of the distances which they travelled were plotted against their presumed molecular sizes, and the mono-, di- and tri-mer were identified by comparison with authentic samples of these purified uronides.

Carboxymethylcellulose. This was prepared as described by Peterson & Sober (1956), and contained 0·5 m-equiv./g.

Buffer solutions. These were formulated according to Gomori (1955), from analytical-grade reagents.

Paper electrophoresis. This was performed on a horizontally supported Whatman no. 1 paper, at 10–20 v/cm., for 20 hr. The protein bands were revealed with bromophenol blue in alcoholic $HgCl_2$ solution (Kunkel & Tiselius, 1951).

pH *measurements.* Where possible these were made with a glass electrode. However, when very small volumes were involved, as in the latter stages of the purification, Special Indicator Papers (E. Merck A.G., Darmstadt, Germany) were used.

Purification of endopolygalacturonase

Fermentation. This was continued for 5 days; Fig. 3 shows the titre of endopolygalacturonase activity, in viscosity-diminishing units, reached at various times during the fermentation. At the end of this period the culture fluid was filtered under vacuum and held at 4° until required.

The following purification scheme describes the quantities of reagents used for 10 l. of culture fluid.

Stage 1. Small portions of the culture fluid were added to various amounts of 0·1 N-$NaHCO_3$, and the pH values of the resultant mixtures were measured with the glass electrode. A titration curve, constructed from the results, was used to calculate the volume of 0·1 N-$NaHCO_3$ required to adjust the bulk of the culture fluid to pH 4·0. The solution was added slowly to the fluid, cooled to 0–2° and stirred mechanically. Cold 95% ethanol (2·5 l.) was added and after 1 hr. the small precipitate was removed. The slow addition of 16·1 l. of cold ethanol raised its concentration to about 65%, and, after the mixture had been kept at −8° to −10° for 2 hr., the precipitated enzyme was harvested by centrifuging.

Stage 2. The precipitate was suspended in 500 ml. of saturated $(NH_4)_2SO_4$ solution at 2°, the resultant volume was measured and the volume of the precipitate estimated by subtracting the 500 ml. volume of $(NH_4)_2SO_4$. For each 100 ml. of precipitate, 50 g. of solid $(NH_4)_2SO_4$ was added. The whole was thoroughly dispersed in a Waring Blendor for 20 min., and then 10 g. of Celite was added and the mixture filtered under vacuum; the residue was resuspended in 125 ml. of 95% saturated $(NH_4)_2SO_4$, stirred for 1 hr. and again filtered through Celite.

The filter cake was resuspended in 250 ml. of 50% saturated $(NH_4)_2SO_4$ and the mixture stirred for 1 hr. and then filtered as before; the residue was once more suspended in 125 ml. of 50% saturated $(NH_4)_2SO_4$, stirred for 1 hr. and filtered.

The combined 50% saturated $(NH_4)_2SO_4$ filtrates were dialysed for 24 hr. against running tap water in a rocking dialyser. A double thickness of Visking cellophan tubing was used as the dialysis membrane, to avoid rupture due to the cellulase present in the preparation.

The material at this stage was reasonably stable and could be stored in solution at 4° for 2–3 weeks.

Stage 3. The last traces of salt were removed from the solution of the enzyme by passing it through a mixed resin column of 40 ml. of Amberlite IR-120 (H^+) and 76 ml. of Amberlite IR-400 (OH^-). The column was washed with 500 ml. of water, which was then added to the eluate. The pH of the mixture was adjusted to 4·2–4·4 by the addition of 0·05 N-acetic acid. Carboxymethylcellulose (6 g.) was suspended in N-acetic acid, filtered off and washed on the filter with three 100 ml. portions of 0·05 M-acetate buffer, pH 4·4. The carboxymethylcellulose was added to the enzyme solution, the mixture was stirred for 30 min. at 4° and then filtered. The residue was washed with two 50 ml. portions of 0·05 M-acetate buffer, pH 4·4, and then suspended in 36 ml. of 0·05 M-acetate buffer, pH 4·6; the pH of the mixture was readjusted to 4·6 by the cautious addition of 0·05 N-sodium acetate. The mixture was stirred for 30 min. and filtered, and the residue washed on the filter with two 36 ml. volumes of 0·05 M-acetate buffer, pH 4·6.

The carboxymethylcellulose was resuspended in 36 ml. of 0·05 M-acetate buffer, pH 5·3, and the pH was adjusted to 5·3 with 0·05 M-sodium acetate. After stirring for 30 min. the mixture was filtered; the carboxymethylcellulose was resuspended in 18 ml. of 0·05 M-acetate buffer, pH 5·3, stirred for 10 min. and filtered, and the filtrate combined with the previous one.

Stage 4. The combined filtrates were treated with small portions of Amberlite resin IR-120 (H^+) until a pH of 4·2–4·4 was obtained. The solution was decanted from the resin, which was washed twice with a little distilled water, the washings being added to the rest of the solution. One gram of carboxymethylcellulose, adjusted to pH 4·4 as before, was added and the mixture stirred for 30 min. The carboxymethylcellulose was filtered off, washed on the filter with two 5 ml. portions of 0·05 M-acetate buffer, pH 4·4, and resuspended in 6 ml. of 0·05 M-acetate buffer, pH 4·6. The mixture was adjusted to pH 4·6 with 0·05 N-sodium acetate, stirred for 30 min. and filtered, and the residue washed on the filter with two 6 ml. portions of 0·05 M-acetate buffer, pH 4·6.

The carboxymethylcellulose was resuspended in 6 ml. of 0·1 M-acetate buffer, pH 5·0, and the mixture was stirred for 30 min. and filtered. The carboxymethylcellulose was again suspended in 3 ml. of 0·1 M-acetate buffer, pH 5·0; the mixture was stirred for 30 min. and filtered, and the filtrate combined with the previous one. The pH of the combined solution was cautiously adjusted to 6·0 with 0·2 M-sodium acetate. This solution constituted the partially purified enzyme preparation used for the experiments described below. Table 1 summarizes the progress of the

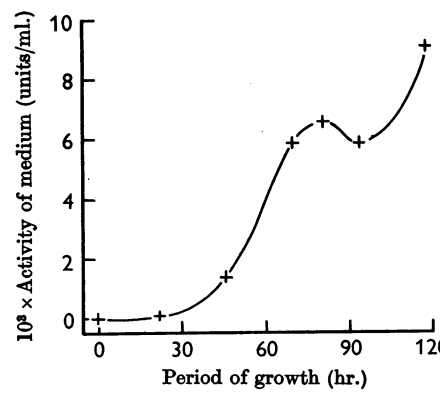

Fig. 3. Production of endopolygalacturonase during the course of a fermentation with *Aspergillus niger*.

Table 1. *Purification of endopolygalacturonase from 6 l. of culture fluid*

Fraction	10^{-3} × Specific activity (units/$E^{1cm.}_{280\,m\mu}$)	Relative activity	10^{-6} × Total activity (units)	Percentage of original activity
Culture fluid	0·61	1	44	100
Stage 1. Fraction pptd. between 20 and 65% (v/v) of ethanol at pH 4·0	2·9	5	35	80
Stage 2. Fraction insoluble in 95% but soluble in 50% saturated $(NH_4)_2SO_4$	15	24	33	76
Stage 3. Fraction eluted from carboxymethylcellulose by 0·05 M-acetate, pH 4·6–5·3	65	100	27	61
Stage 4. Fraction eluted from carboxymethylcellulose by acetate between 0·05 M, pH 4·6, and 0·1 M, pH 5·0	280	400	15	34

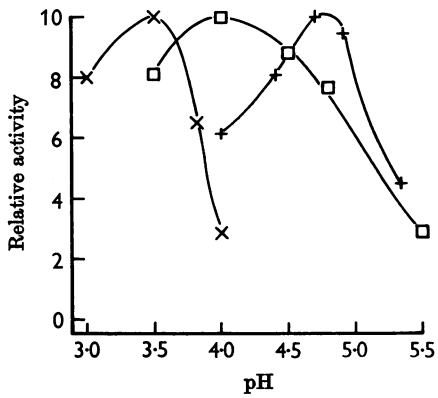

Fig. 4. pH optima for the activity of endopolygalacturonase measured in terms of (*a*) the diminution in viscosity of a 1% solution of pectic acid (+), (*b*) the release of reducing groups from a 0·5% solution of pectic acid (□), (*c*) the release of reducing groups from a 0·5% solution of trigalacturonic acid (×).

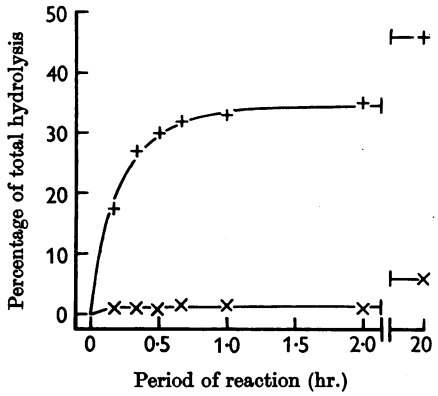

Fig. 5. Hydrolysis of 1% solutions of pectin (×) and pectic acid (+), measured by the release of reducing groups at pH 4·0.

purification. The final preparation contained about 35 mg. of protein on an extinction basis with a specific activity of some $8·8 \times 10^5$ viscosity-diminishing units/mg. of protein. Numerous preparations were performed over a period of a year without failure.

RESULTS

Paper electrophoresis. In phosphate buffers of pH 7·2 and 8·0, and ionic strength 0·15, the partially purified preparation gave a single sharply defined band on paper electrophoresis.

Action of the enzyme against pectin and pectic acid

Reduction of viscosity. The enzyme produced a rapid fall in the viscosity of solutions of pectin and pectic acid at pH 4·8. However, the preparation was 2·7 times as active, in this respect, when acting on pectic acid as on pectin.

To determine the optimum pH for the action of the enzyme against pectic acid, 0·5 ml. portions of 2% sodium pectate at the desired pH values were mixed with 0·5 ml. of dilutions of the enzyme in 0·2 M-acetate buffers of the same pH values and the fall in viscosity followed in the normal manner; the pH values of the digests were then measured. The enzyme showed its maximum activity at pH 4·7–4·8 (Fig. 4*a*).

Release of reducing groups. There were marked differences from the previous results when the activity of the enzyme was followed by measuring the release of reducing groups; pectic acid rapidly undergoes a 30–40% hydrolysis but pectin is little affected (Fig. 5). The optimum pH for the release of reducing groups from pectic acid was determined by mixing 4 ml. of 1% sodium pectate, preadjusted to the desired pH, 3 ml. of 0·2 M-acetate buffer, at the same pH, and 1 ml. of a suitable dilution of the enzyme in water. After incubation for 10 min. at 30°, the reaction was stopped by adding 2 ml. of 2 N-Na_2CO_3, and the reducing groups were determined by the hypoiodite method. Starting blanks were obtained by adding the ingredients to flasks containing the 2 N-Na_2CO_3. An optimum for the reaction was obtained at pH 4·0–4·2 (Fig. 4*b*). Enzyme concentrations were adjusted so that all

determinations were made within the initial region of rapid hydrolysis.

The breakdown of pectic acid was very rapid initially but became very slow after 40% hydrolysis has been obtained; nevertheless, in the presence of very high enzyme concentrations it will continue to at least 60%. With a digestion mixture containing 0·5% of pectic acid and 6000 viscosity-diminishing units of enzyme/ml., the level of digestion had reached 35% after 2 min. and 41, 54 and 61% after 44, 145 and 185 hr. respectively.

The initial stages of pectic acid hydrolysis were followed by spotting on chromatographic paper 0·01 ml. portions of a reaction mixture of 0·5% sodium pectate and 60 viscosity units of enzyme/ml., after various short periods of reaction. The spots were dried immediately in a stream of air, and then the paper was developed in the butyric acid–acetic acid–water solvent. The sample taken after digestion for 2 min. showed faint spots corresponding to mono-, tri-, tetra-, penta- and hexa-galacturonic acid; these were joined by the digalacturonic acid spot in the 4 min. sample. The intensities of all the spots now increased with increasing reaction time up to the final sample at 30 min. When higher enzyme concentrations and longer reaction times were used in order to make a similar examination of the later stages of the reaction, the spots corresponding to the higher galacturonides progressively disappeared with increasing reaction time. The disappearance of the trigalacturonic acid spot was very slow and the digalacturonic acid spot remained.

Action of the enzyme against purified oligogalacturonides

The action of the enzyme against tetra-, tri- and di-galacturonic acid was followed chromatographically by mixing 0·5% solutions of the neutralized acids with suitable concentrations of enzyme and spotting samples of the mixture on paper as described for the pectic acid. Tetragalacturonic acid underwent a fairly rapid split to tri- and mono-galacturonic acid, and trigalacturonic acid underwent a much slower split to di- and mono-galacturonic acid. Digalacturonic acid, however, was not attacked.

The actual rate of breakdown of trigalacturonic acid was measured by the colorimetric hypoiodite method, and it was confirmed that the digalacturonic acid was resistant to hydrolysis by this enzyme; Table 2 compares the rates of hydrolysis of pectic acid (at pH 4·0) and tri- and di-galacturonic acid (at pH 3·5) under the influence of the enzyme preparations stage 1 and stage 4 used at equivalent viscosity-diminishing activities.

The optimum pH for the hydrolysis of trigalacturonic acid by the enzyme preparation stage 4 was found by measuring the rate of hydrolysis of this substrate by the colorimetric hypoiodite method, with, as reaction mixtures, 0·4 ml. of each of a suitable range of 0·2 M-buffers, 0·1 ml. of a suitable dilution of the enzyme in water and 0·5 ml. of a neutralized 1% solution of trigalacturonic acid. Maximum activity was at pH 3·5 (Fig. 4*c*).

Stability of endopolygalacturonase

The effect of pH on stability of the enzyme at 50° and 80° was investigated by diluting a solution of the enzyme to 1000 viscosity-diminishing units/ml. with each of a range of 0·1 M-citrate–phosphate buffers. A portion (3 ml.) of each dilution, the pH of which was checked, was then placed in stoppered tubes and heated for exactly 10 min. in a large water bath held at the desired temperature. The tubes were quickly cooled and a small portion of

Table 2. *Relative rates of hydrolysis of different substrates by pectic enzyme preparations*

Figures are given for the rates of hydrolysis of the substrates shown with enzyme preparations stage 1 and stage 4. Hydrolyses were performed at 30° with 0·5% substrate concentration throughout.

Substrate	pH	PGu/10⁶ viscosity-diminishing units: Stage 1	Stage 4
Pectic acid	4·0	10·3	8·0
Trigalacturonic acid	3·5	0·065	0·009
Digalacturonic acid	3·5	0·022	0·00

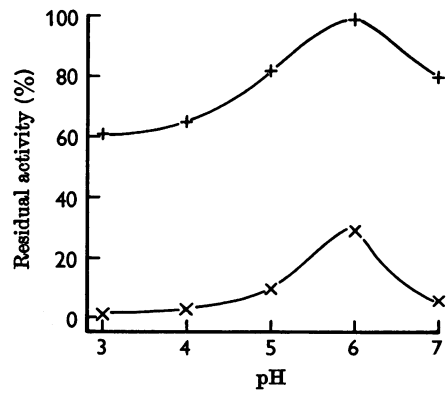

Fig. 6. Effects of pH and temperature on the stability of endopolygalacturonase in a solution containing 1000 viscosity-diminishing units/ml. Samples at the various pH values shown were held at 50° (+), and at 80° (×), for 10 min.

their contents was withdrawn and immediately diluted with 0·2 M-acetate buffer, pH 4·8, before assay by the viscosity method. A narrow range of enhanced stability was found about pH 6 (Fig. 6).

When stored at 5° and pH 6 the preparation stage 4 lost some 15 % of its activity in 5 days. Attempts to increase the stability by the addition of pectin, sucrose, galacturonic acid, gelatin, BAL (2:3-dimercaptopropanol), cysteine or ethylenediaminetetra-acetic acid were uniformly unsuccessful. The preparation lost much of its activity when air was bubbled through it or when it was frozen and thawed.

DISCUSSION

The method of purification described here has resulted in an approximately 400-fold purification of the endopolygalacturonase from culture fluids of *A. niger*. The only other clear investigation of this enzyme from this source which could be found in the literature is that of Saito (1955). This author selected a unit of viscosity-diminishing activity such that 10 units would diminish the viscosity of 5 ml. of a 0·8 % solution of pectic acid by 50 % in 1 hr. at pH 4·2 and 30°. It is especially difficult to compare the results of viscosity assays by authors using different types of pectic acids (Saito used a citrus pectic acid with a uronic anhydride content of only 69 %), but so far as such a comparison is valid it would appear that one of our units of activity is equivalent to about 12 of Saito's. His purified material had an activity of 3000 of his units per mg. (equivalent to 250 of our units per mg.). Our preparations are thus more than 1000-fold as active as those of Saito.

Lineweaver, Jang & Jansen (1949) purified a polygalacturonase of unspecified type from the commercial preparation Pectinol, obtaining an activity of 1·41 PGu/mg. of total nitrogen. This suggests a value of 0·23 PGu/mg. of protein. Our preparations have activities of more than 2 PGu/mg. of protein.

The pattern of production of oligogalacturonides during the hydrolysis of pectic acid by the enzyme shows that there is a random mechanism of attack of this substrate which, taken with the high activity found in the viscosity assay, clearly demonstrates that an 'endo' enzyme is involved. The enzyme will rapidly reduce the viscosity of a pectin solution but the activity is slower than that with pectic acid. Reid (1952) calculated that the scission of only 0·5–1 % of the internal glycosidic bonds of pectic substances would reduce the viscosity of their solution by 50 %. Since very nearly half of the carboxylic groups in our pectin remain unesterified it is not surprising that some length of the polymer should be susceptible to an endopolygalacturonase, i.e. to an enzyme which preferentially attacks the bond linking two unesterified galacturonic acid residues. Moreover, the very slight release of reducing groups which the enzyme brings about from pectin makes it clear that an endopolygalacturonase is indeed involved.

The fall in the noted optimum pH values which we found in the viscosity assay, the release of reducing groups from pectic acid and the release of the reducing groups from trigalacturonic acid are consistent with the observation of Demain & Phaff (1954) and Saito (1955) that the smaller the substrate size the lower was the optimum pH; in the three assays mentioned the mean substrates are respectively pectic acid, high-molecular-weight oligouronides produced by 5–10 % hydrolysis of pectic acid, and the low-molecular-weight trigalacturonic acid. The values of 4·7–4·8 for the viscosity assay and 4·0–4·2 for the reducing-group assay are in good agreement with those reported by Saito.

The question of the range of substrate sizes attacked by endopolygalacturonase is particularly difficult. The endopolygalacturonase of the yeast *S. fragilis* will attack all of the galacturonides except digalacturonic acid (Demain & Phaff, 1954; Phaff & Demain, 1956; Patel & Phaff, 1959). Brooks & Reid (1955) found that a similar enzyme from *Aspergillus foetidis* could not attack either di- or tri-galacturonic acid. Saito's (1955) enzyme, also, did not apparently attack these substances. But McCready & Seegmiller (1954) reported that their enzyme from Pectinol attacked both these uronides at a rate about 5 % of that at which it attacked pectic acid.

Our preparation stage 4 induces a slow but definite hydrolysis of trigalacturonic acid but is completely devoid of action against digalacturonic acid. However, one single preparation of stage 4 did produce a slight hydrolysis of digalacturonic acid; this result could not be repeated with other batches and was doubtless due to the presence of a contaminating enzyme in the preparation. That preparation stage 1 contains another pectic enzyme is clear, in that this preparation splits digalacturonic acid at an appreciable rate and trigalacturonic acid at a much higher rate than does preparation stage 4.

The behaviour of *A. niger* endopolygalacturonase is thus analogous with that of *S. fragilis*; Demain & Phaff (1954) postulated that one of the terminal residues of the galacturonide chain protected the adjacent glycosidic bond completely, and the next glycosodic bond partially, against attack by yeast endopolygalacturonase. Apart from this proviso all the other bonds of the chain were supposed to be freely susceptible to attack. This hypothesis satisfactorily explains the action of the *A. niger* enzyme also.

SUMMARY

1. Endopolygalacturonase has been separated from the culture fluid of *Aspergillus niger* with a purification factor of 400-fold and a recovery of 34%.

2. The preparation obtained was homogeneous on paper electrophoresis at pH 7·2 and pH 8·0, and of moderate stability with an optimum for stability of about pH 6.

3. The enzyme rapidly lowered the viscosity of a solution of pectic acid and released reducing groups from it according to a random pattern of hydrolysis, but the digestion was not complete. Pectin underwent only a very slight degradation, sufficient to show a fall in viscosity but with little release of reducing groups.

4. Tetragalacturonic acid was rapidly split to tri- and mono-galacturonic acid, and trigalacturonic acid was slowly split to di- and monogalacturonic acid, but the digalacturonic acid was not attacked.

We wish to acknowledge the provision of facilities and research fellowships by the Istituto Superiore di Sanità. We are especially grateful to Professor E. B. Chain, F.R.S., under whose direction this work was performed. Thanks are due to Mr K. L. Wilford for skilled technical assistance.

REFERENCES

Ashby, J., Brooks, J. & Reid, W. W. (1955). *Chem. & Ind.* p. 360.
Brooks, J. & Reid, W. W. (1955). *Chem. & Ind.* p. 325.
Demain, A. L. & Phaff, H. J. (1954). *J. biol. Chem.* **210**, 381.
Demain, A. L. & Phaff, H. J. (1957). *Wallerstein Labs Commun.* **20**, 119.
Dixon, M. & Webb, E. C. (1958). In *Enzymes*, p. 39. London: Longmans, Green and Co. Ltd.
Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 138. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Kertesz, Z. I. (1951). In *The Pectic Substances*, p. 128. New York: Interscience Publishers Inc.
Kunkel, H. G. & Tiselius, A. (1951). *J. gen. Physiol.* **35**, 89.
Lineweaver, H., Jang, R. & Jansen, E. F. (1949). *Arch. Biochem.* **20**, 137.
Luh, B. S. & Phaff, H. J. (1954). *Arch. Biochem. Biophys.* **51**, 102.
McCready, R. M. & Seegmiller, C. G. (1954). *Arch. Biochem. Biophys.* **50**, 440.
McCready, R. M., Swenson, H. A. & Maclay, W. D. (1946). *Industr. Engng Chem. (Anal.)*, **18**, 290.
Myers, P. B. & Baker, G. L. (1934). *Bull. Del. agric. Exp. Sta.* no. 187.
Owens, H. S., McCready, R. M., Shepheard, A. D., Schultz, T. H., Puppen, E. L., Swenson, H. A., Miers, J. C., Erlandsen, R. F. & Maclay, W. D. (1952). In *Methods Used at the Western Regional Research Laboratory for Extraction and Analysis of Pectic Material, Circ. U.S. Dep. Agric.*, AIC 340.
Patel, D. S. & Phaff, H. J. (1959). *J. biol. Chem.* **234**, 237.
Peterson, E. A. & Sober, H. A. (1956). *J. Amer. chem. Soc.* **78**, 751.
Phaff, H. J. & Demain, A. L. (1956). *J. biol. Chem.* **218**, 875.
Reid, W. W. (1952). *Biochem. J.* **50**, 289.
Roboz, E., Barrett, R. W. & Tatum, E. L. (1952). *J. biol. Chem.* **195**, 459.
Saito, H. (1955). *J. gen. appl. Microbiol.* **1**, 38.
Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
Tuttobello, R. & Mill, P. J. (1961). *Biochem. J.* **79**, 51.

Biochem. J. (1961) **79**, 64

Chemical Reactions which Affect the Biological Activity of Human Gonadotrophins

BY W. R. BUTT, A. C. CROOKE, F. J. CUNNINGHAM AND ANNE JAMESON EVANS
The United Birmingham Hospitals Department of Clinical Endocrinology, Birmingham, 11

(*Received* 8 *August* 1960)

The biological activity of the gonadotrophic hormones is diminished by the action of various reagents. The effects of oxidation, reduction, acetylation and enzymic hydrolysis have been extensively investigated and certain differences in the relative stabilities of follicle-stimulating hormone (FSH) and interstitial-cell-stimulating hormone (ICSH) have been reported.

The gonadotrophins are glycoproteins and the carbohydrate groups appear to be important for biological activity. Thus Whitten (1950) demonstrated that the activity of ICSH in serum gonadotrophin was retained after treatment with 2 mM-periodate but the activity of FSH was diminished. Geschwind & Li (1958), however, claimed that both gonadotrophins prepared from the anterior-pituitary glands of sheep were destroyed by treatment with 0·2 M-potassium periodate.

Fraenkel-Conrat, Simpson & Evans (1939, 1940) and McShan & Meyer (1940) showed that reduction