- McCollum, E. V., Simmonds, N. & Pitz, W. (1917). *J. biol. Chem.* **29**, 521.
- Macpherson, H. T. (1946). *Biochem. J.* **40**, 470.
- Neuberger, A. & Sanger, F. (1942). *Biochem. J.* **36**, 662.
- Osborne, T. B. & Campbell, G. F. (1895). *Rept. Conn. agric. Exp. Sta.*
- Osborne, T. B. & Campbell, G. F. (1896). *J. Amer. chem. Soc.* **18**, 575.
- Rees, M. W. (1946). *Biochem. J.* **40**, 632.
- Rose, M. S. & Cooper, L. F. (1917). *J. biol. Chem.* **30**, 201.
- Rose, W. C., Oesterling, M. J. & Womack, M. (1948). *J. biol. Chem.* **176**, 753.
- Rubner, M. (1879). *Z. Biol.* **15**, 115.
- Rubner, M. & Thomas, K. (1918). *Archiv. Physiol. Lpz.* (Abt. Physiol.), p. 1.
- Slack, E. B. (1948a). *Nature, Lond.*, **161**, 211.
- Slack, E. B. (1948b). *Brit. J. Nutrit.* **2**, 214.
- Stutzer, A. (1880). *J. Landw.* **28**, 103.
- Tristram, G. R. (1946). *Biochem. J.* **40**, 721.
- Vickery, H. B. (1925). *J. biol. Chem.* **65**, 657.

The Pectic Enzymes of the Mould *Byssoschlamys fulva*

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The fungus *Byssoschlamys fulva* was discovered by Olliver & Smith (1933), and its action on pectic substances first studied by Olliver & Rendle (1934). Hirst & McMaster (1932-3); Hull (1933-4, 1934-5, 1939) and Gillespy (1936-7, 1939, 1940, 1946) have described its characteristics, while its metabolic products have been investigated by Raistrick & Smith (1933). It derives its economic importance from three characteristics, two of which are unusual or even unique: (a) its ascospores have an abnormally high heat resistance and can readily withstand the normal processes used in bottling fruit, though only a small proportion survive the higher temperatures used in canning, (b) it can grow under conditions of greatly reduced oxygen tension, e.g. in canned and bottled fruit, and (c) it secretes pectic enzymes which can cause disintegration of the fruit tissue. Ascospores of *B. fulva* are thus liable to survive the usual bottling processes and cause more or less complete breakdown of the fruit.

The present work is an attempt to identify the particular pectic enzymes involved. The nomenclature used in this paper is in conformity with current views on the structure of pectin (Hirst & Jones, 1946), which support the classification of pectic enzymes proposed by Kertesz (1936): (1) protopectinase (or pectosase) which converts protopectin into soluble pectin; (2) pectin methoxylase (or pectase) which converts pectin into pectic acid and methanol; and (3) pectinase (probably a group of enzymes) which causes a reduction in the molecular size of the pectin and leads ultimately to D-galacturonic acid. The group contains at least a disaggregating enzyme and polygalacturonase.

EXPERIMENTAL

Enzymes. Three sources were used: (1) bottled fruit which showed pronounced disintegration due to infection with

B. fulva, the fruit juice being separated from the disintegrated fruit tissues by repeated filtration through cotton wool; (2) Czapek-Dox solution on which a pure culture of the mould had been grown; (3) extracts of the fungal mycelium collected from various types of culture solution. More concentrated enzyme preparations were obtained from each of these sources by the addition of ethanol (3 vol. to 1 vol. of solution) and separation of the flocculent precipitate in a centrifuge. The solid product was freed from ethanol by drying over CaCl₂ in an evacuated desiccator and then dissolved in a small volume of water. The solution was kept sterile by storage at low temperature over CHCl₃.

Substrates. Potato disks of standard dimensions (1.2 cm. diam., 0.5 mm. thick) prepared on a simple hand microtome were used for the protopectinase tests. Pectin solutions (prepared from citrus pectin, 100 grade, British Drug Houses Ltd.) and polygalacturonide methyl ester, prepared by the action of methanolic HCl on pectin (Morell, Baur & Link, 1934) were used for the pectin methoxylase and pectinase tests. Pectic acid, prepared by demethoxylating pectin with pectin methoxylase from tomatoes, was also used for some of the pectinase tests.

Methods employed for enzyme detection. (a) Protopectinase was detected by its macerating action on thin disks of potato tuber at 37°. The disks were periodically removed from the enzyme solution and any maceration observed by gently pulling them with blunt-ended forceps. The results were placed on a semi-quantitative basis by using a scale of softening ranging from 'just detectable' (+) to 'complete disintegration' (+++). (b) Pectin methoxylase activity was examined by Kertesz's (1937) method in which the carboxylic acid groups set free by the action of the enzyme are titrated with dilute alkali, using methyl red as indicator. (c) Pectinase activity is difficult to assess because of the complexity of the reactions involved. Periodic determinations of the viscosity and of the reducing power of pectin solutions on treatment with the enzyme preparation were chosen as measuring respectively the concentrations of unchanged substrate and the final decomposition product, D-galacturonic acid.

Blank experiments with heat-inactivated enzyme solutions were included in all tests.

RESULTS

Protopectinase. Protopectinase activity was detected in four out of seven cultures of *B. fulva* in Czapek-Dox medium all containing 5 % glucose, and in some cases, 0.05 % asparagine. The most active culture gave + and ++ ratings in the potato disk test in 3 and 18 hr. respectively. Two of those inactive were anaerobic cultures with submerged flocculent mycelia, in contrast to the thick white surface mycelia observed in aerobic cultures at pH 3-4. The single inactive aerobic culture was grown at pH 5 and formed a partly submerged mycelium similar to the anaerobic cultures. There was some indication that the addition of asparagine to the culture medium enhanced the production of protopectinase.

Protopectinase activity was detected in five out of six specimens of bottled fruit (plums, damsons, gooseberries) showing partial or complete disintegration of the fruit tissue. The most active specimen gave a potato disk rating of ++ in 6 hr., the remaining four showing +++ in 24 hr.

Activity tests over the pH range 2-8 were made with filtered fruit juice, the pH of the juice being adjusted with N-acid or alkali. Maximum activity was observed at pH 4-5 (cf. Davison & Willaman, 1927; Brown 1915, who gave pH 5 and 6 respectively).

Experiments on the thermal inactivation of the enzyme at pH 4 are shown in Table 1. The results indicate that the enzyme is completely inactivated in a medium of pH 4 by exposure to a temperature of 65° for 5-10 min.

Table 1. *Effect of heat on protopectinase of Byssoschlamys fulva*

Temp. (°)	Time of heating (min.)			
	5	10	15	20
	Activity rating (see test)			
50	++	++	++	++
55	++	++	++	++
60	++	++	++	+
65	±	-	-	-
70	-	-	-	-

Pectin methoxylase. This enzyme was consistently absent from *B. fulva* culture media, from extracts of the fungal mycelium, and from the juice of infected bottles of fruit. The specimens tested were the same as those used in the protopectinase tests.

Control experiments with tomato juice and the commercial pectic enzyme preparation known as 'Pectinol 10 M,' using 1 % pectin solutions as substrate, showed that Kertesz's (1937) method is only suitable for the qualitative demonstration of pectin methoxylase in an enzyme system. Quantitative tests are made difficult by an apparent dependence

of the activity of the enzyme sample (in 'Kertesz units'/ml.), on the size of the sample and the duration of the test. In a typical experiment a 1 % pectin solution (40 ml.), which had been adjusted to pH 6.5 with 0.1 N-sodium hydroxide using methyl red as indicator, was treated with 1 ml. of a 'Pectinol 10 M' solution containing 0.2 mg. of the enzyme; acid was formed in the solution in 2.5 hr. at 15°. In similar experiments, using *B. fulva* enzyme preparations in various concentrations (1-5 ml. of the concentrated solution in 40 ml. of 1 % pectin solution), no acid was produced, even after 5 days. Further experiments at 30° again pointed to the absence of pectin methoxylase activity in the *B. fulva* enzyme system, whereas at this temperature 'Pectinol 10 M', in the same concentration as before, produced acid within 1 hr.

Independent pH measurements using a Cambridge pH meter confirmed the failure of all the *B. fulva* enzyme preparations to produce acid from a pectin substrate.

Pectinase complex. Pectin solutions which were treated with *B. fulva* enzyme preparations at 25 and 37° showed a rapid initial fall in viscosity, followed by a slower fall to a constant value (see Fig. 1).

Fig. 1. Effect of *B. fulva* enzymes on the viscosity of 1 % pectin solution at 25°.

Simultaneous determinations of the reducing power by the alkaline iodide method (Willstätter & Schudel, 1918; Ingles & Israel, 1948) indicated that only a minute increase took place which was of the same order as the experimental error of the estimation (0.05 ml. of 0.1 N-iodine). Typical results are shown in Table 2.

The results obtained over the pH range 3-5 were quantitatively similar and an optimum pH value was not apparent.

Control experiments with 'Pectinol 10 M' at 37° indicated that the reducing power of 0.4 % pectin solution did not increase until the specific viscosity of the solution had fallen to a value of approxi-

mately 0.2. Further tests were thus made with a *B. fulva* enzyme preparation in which the pectin solutions were held for long periods at 37° after the viscosity had reached a constant value ($\eta_{sp}^{25} = 0.12$). Even 1000 hr. after the viscosity of the pectin solution had become constant, the increase in reducing power remained negligible. On the other hand, 0.4 % pectin solution (40 ml.) which had been treated with 0.2 mg. of 'Pectinol 10 M' at 37° rapidly gave an increase in reducing power equivalent to 0.29 ml. of 0.1 N-iodine/2 ml. of pectin solution.

Table 2. *Effect of Byssochlamys fulva enzymes on the viscosity and reducing power of pectin*

Pectin (%)	Mixture	Temp. (°)	Viscosity (η_{sp}^{25})		Reducing power of 2ml. mixture (ml. 0.1N-I ₂)	
			Initial	Final	Initial	Final
1.0	Test	25	2.22	0.70	0.13	0.15
1.0	Blank	25	2.22	1.99	0.10	0.13
0.4	Test	37	1.31	0.12	0.33	0.38
0.4	Blank	37	1.31	1.02	0.34	0.39

The material recovered by precipitation with ethanol from the *B. fulva* test and from blank solutions after the viscosities had become constant showed markedly different physical properties. The precipitate from the test solution was non-gelatinous and flocculent, whereas that from the blank solution was gelatinous and voluminous, resembling un-degraded pectin precipitated under the same conditions.

A 1 % polygalacturonide methyl ester solution ($\eta_{sp}^{25} = 0.27$) was treated with an extract of *B. fulva* mycelium which rapidly disaggregated pectin. The viscosity of the solution did not fall in 300 hr. and the reducing power of the polyester was similarly unaffected.

In view of the fact that Jansen & MacDonnell (1945) have stated that only de-esterified pectic substances are attacked by polygalacturonase, a suspension of pectic acid in water was treated with the *B. fulva* enzymes. Failure to obtain any increase in reducing power further confirmed the absence of polygalacturonase from the *B. fulva* enzyme system.

DISCUSSION

The present results show that *B. fulva* produces an enzyme system possessing protopectinase and pectinase activity. Protopectinase activity is found in many fungi and bacteria (Branfoot, 1929) and it has long been recognized that the macerating action of many plant pathogens is due to the production of protopectinase and the consequent destruction of the middle lamella. Protopectin, the substrate for

this enzyme, is ill defined so that work on this enzyme has been mainly qualitative.

Although little attention has been paid to the individual components of the complex, the pectinase group of enzymes appears to contain at least polygalacturonase and a disaggregating enzyme. Many workers have obtained pectinase preparations with very marked polygalacturonase activity; thus Ehrlich (1933, 1935), Mottern & Cole (1939), and Manville, Reithel & Yamada (1939) have broken down pectin completely into D-galacturonic acid. On the other hand, Kertesz (1939) has observed that many pectinase preparations from various *Penicillia* bring about the development of only a small fraction of the maximum possible reducing power from pectin. This variation in activity may be due to the existence of two or more polygalacturonases, acting on pectic substances of various molecular sizes, so that all the enzymes are necessary to bring about the complete sequence of reactions required to produce D-galacturonic acid. Kertesz (1939) has suggested that two different types of enzyme are involved in pectinase activity; the first disaggregating the pectin molecule, with a consequent fall in viscosity, and the second attacking the product so formed, opening the glycosidic bonds between the uronic acid residues with a consequent increase in reducing power.

The results of the present investigation indicate that a very large reduction in the molecular size of pectin is brought about by the enzyme system of *B. fulva*, without any increase in reducing power, and therefore without any fission of glycosidic bonds. This result is an extreme case of the phenomenon noted by Kertesz (1939) amongst the pectinases of various *Penicillia*. It is considered that the results provide confirmatory evidence for Kertesz's view that the large molecular size of pectin is mainly due to the existence of aggregates, consisting of relatively small polyuronic acid units joined by linkages which are not glycosidic but possibly of the nature of hydrogen bonds.

SUMMARY

1. The enzyme system produced by the mould *Byssochlamys fulva* has been examined. Evidence has been found for the presence of protopectinase and a disaggregating enzyme which reduces the molecular size of pectin without the production of galacturonic acid.

2. The production by a fungus of a pectinase type of enzyme which is free from pectin methoxylase has not been reported hitherto.

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REFERENCES

- Branfoot, M. H. (1929). Food Investigation Board, Special Rep. no. 33. London: H.M.S.O.
- Brown, W. (1915). *Ann. Bot., Lond.*, **29**, 313.
- Davison, F. R. & Willaman, J. J. (1927). *Bot. Gaz.* **83**, 329.
- Ehrlich, F. (1933). *Biochem. Z.* **259**, 100.
- Ehrlich, F. (1935). *Biochem. Z.* **281**, 93.
- Gillespy, T. G. (1936-7). *Rep. Fruit Veg. Pres. Sta., Campden*, p. 68.
- Gillespy, T. G. (1939). *Rep. Fruit Veg. Pres. Sta., Campden*, p. 68.
- Gillespy, T. G. (1940). *Rep. Fruit Veg. Pres. Sta., Campden*, p. 54.
- Gillespy, T. G. (1946). *Rep. Fruit Veg. Pres. Sta., Campden*, p. 31.
- Hirst, E. L. & Jones, J. K. N. (1946). *Advances in Carbohydrate Chemistry*, **2**. New York: Academic Press.
- Hirst, F. & McMaster, N. B. (1932-3). *Rep. Fruit Veg. Pres. Sta., Campden*, p. 53.
- Hull, R. (1933-4). *Rep. Fruit Veg. Pres. Sta., Campden*, p. 64.
- Hull, R. (1934-5). *Rep. Fruit Veg. Pres. Sta., Campden*, p. 65.
- Hull, R. (1939). *Ann. appl. Biol.* **26**, 800.
- Ingles, O. G. & Israel, G. C. (1948). *J. chem. Soc.* p. 810.
- Jansen, E. F. & MacDonnell, L. R. (1945). *Arch. Biochem.* **8**, 97.
- Kertesz, Z. I. (1936). *Ergebn. Enzymforsch.* **5**, 233.
- Kertesz, Z. I. (1937). *J. biol. Chem.* **121**, 589.
- Kertesz, Z. I. (1939). *J. Amer. chem. Soc.* **61**, 2544.
- Manville, I. A., Reithel, F. J. & Yamada, P. M. (1939). *J. Amer. chem. Soc.* **61**, 2973.
- Morell, S., Baur, L. & Link, K. P. (1934). *J. biol. Chem.* **105**, 1.
- Mottern, H. H. & Cole, H. L. (1939). *J. Amer. chem. Soc.* **61**, 2701.
- Olliver, M. & Rendle, T. (1934). *J. Soc., chem. Ind., Lond.*, **53**, 166T.
- Olliver, M. & Smith, G. (1933). *J. Bot., Lond.*, **71**, 196.
- Raistrick, H. & Smith, G. (1933). *Biochem. J.* **27**, 1814.
- Willstätter, R. & Schudel, G. (1918). *Ber. dtsh. chem. Ges.* **51**, 780.

Studies on Suramin (Antrypol: Bayer 205)

7. FURTHER OBSERVATIONS ON THE COMBINATION OF THE DRUG WITH PROTEINS

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Little is known about the metabolic fate of injected suramin, the trypanocidal drug which is retained in the body for several weeks or even months after its injection. Even before satisfactory methods for the determination of suramin became available, however, it was suggested that the injected drug combines with the body proteins (for a review of the early literature see Findlay, 1939), and this view received support from the observation that the addition of suramin effects marked changes in some of the properties of certain proteins; for example, the serum proteins are protected against heat coagulation (Collier, 1926, 1927) and against precipitation by tannin, mercuric chloride and certain other reagents (Jirovec & Kocian, 1930; Kocian, 1936), the isoelectric points of some immune substances are altered (Klopstock, 1932), and the drug inhibits a few enzymes, e.g. trypsin (Beilinson, 1929), fumarase (Quastel, 1931) and hyaluronidase (Beiler & Martin, 1948).

Quantitative measurement of the amount of suramin which combines under varying conditions with the plasma proteins of animals injected with the

drug, or in experiments *in vitro* with various proteins, became possible when a satisfactory chemical method became available for the determination of the drug (Dangerfield, Gaunt & Wormall, 1938). Using this method it was conclusively shown that suramin can combine with many different proteins (serum globulin, fibrinogen, casein, gelatin) under physiological conditions of pH and temperature (Bournsnel & Wormall, 1939; Dewey & Wormall, 1946).

At present we are investigating the action of suramin on enzymes. It was thought, therefore, that a study of the conditions under which the drug combines with proteins, and a further investigation of the nature of the linkages concerned, might throw some light on the specific inhibitory effect which the drug exerts on some enzyme systems. It was also hoped that useful information might be obtained about the mechanism whereby suramin inactivates complement and the blood-clotting system.

The present paper describes a study of (i) the stability of the linkage between the drug and various proteins, (ii) the influence of pH on the combination,