Phytochemistry, 1970, Vol. 9, pp. 2447 to 2452. Pergamon Press. Printed in England.

THE USE OF A FUNGAL PECTATE LYASE IN THE PURIFICATION OF LACCASE FROM PEACHES

E. HAREL and A. M. MAYER

Botany Department, The Hebrew University, Jerusalem, Israel

(*Received* 17 *February* 1970)

Abstract—The purification of laccase from peaches is hampered by the presence of large amounts of pectic substances in the fruit. The pectic substances can be removed by the use of pectate lyase, purified from a crude preparation of pectolytic enzymes (Pectinol R-10) which is commercially available. This method may be of general use in purifying enzymes from plant tissues rich in pectic substances.

INTRODUCTION

WE HAVE previously reported that peaches contain at least two enzymes capable of oxidizing phenolic compounds.[1,2] One is present in a particulate fraction of the cell and is of the catechol oxidase type (E.C. 1.10.3.1). The second is a laccase-like enzyme (*p*-diphenol oxygen-oxidoreductase E.C. 1.10.3.2). The latter is present exclusively in the soluble fraction, while the catechol oxidase can apparently leach from the particulate to the soluble fraction.[2]

A major difficulty in purification of the laccase-like enzyme (and of other enzymes from the soluble fraction of many fruits in general) is the presence of large amounts of pectic substances in the fruit. These substances clog up columns and tend to co-precipitate with proteins on ammonium sulphate or acetone fractionation. Partial purification and characterization of the laccase-like enzyme has been reported.[2] However, the method employed, based on removal of the pectic material by precipitation with quaternary aliphatic ammonium salts, involved laborious and lengthy procedures. Ways were therefore sought to simplify the purification procedure.

RESULTS AND DISCUSSION

Insoluble polyvinyl pyrrolidone (Polyclar AT) has been used for the removal of phenols during the separation of subcellular fraction and in the initial steps of the purification of enzymes from plant tissues.[3] It was found to improve the integrity of the isolated particles and protect enzymes from inactivation.[3] We have included Polyclar AT (prewashed to remove soluble material) in the initial step of purification after it was found that Polyclar, up to 1 g/10 g destoned peaches, increased the activity of laccase recovered in the supernatant by 25%. The increase in catechol oxidase activity was 50–70%. A further increase (2·5–3·0-fold) in the activity of both enzymes was obtained after precipitation in 60% cold acetone. This might suggest that not all the endogenous phenols were removed by the Polyclar.

Attempts at removing pectic material by precipitation as calcium salts, either before or after alkaline hydrolysis, were unsuccessful. The pectic material was precipitated as a gel

[1] A. M. MAYER, E. HAREL and Y. SHAIN, *Phytochem.* 3, 447 (1964).
[2] A. M. MAYER and E. HAREL, *Phytochem.* 7, 1253 (1968).
[3] W. D. LOOMIS and J. BATTAILE, *Phytochem.* 5, 423 (1966).

when increasing amounts of calcium nitrate were added to the peach preparation in tris-maleate buffer pH 6·0. The activities of both laccase and catechol oxidase in the supernatant also decreased, the loss of enzyme activity following closely the removal of pectic material from solution.

Pectinol R-10 (Rohm & Haas) contains a pectate lyase (E.C. 4.2.99.3) of fungal origin, bound to kieselguhr.[4] It was decided to try to use it in order to remove the pectic substances from the peach preparation. 1 g of Pectinol R-10 was suspended in 6 ml water, brought to pH 8·3 with NaOH, centrifuged at 20,000 *g* for 15 min and the supernatant filtered through

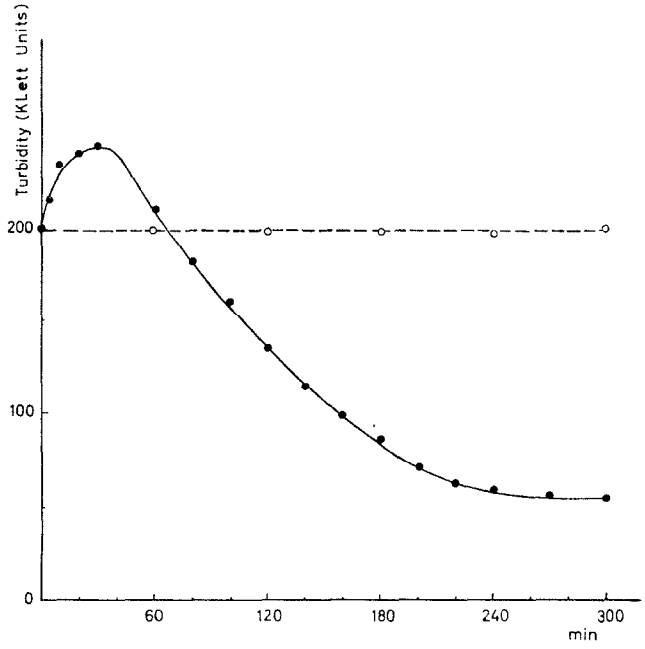

FIG. 1. DECOMPOSITION OF COMMERCIAL PECTIN ON INCUBATION WITH PECTATE LYASE FROM PECTINOL R-10.

The reaction mixture consisted of 8·5 ml 0·6% pectin in 0·5 M acetate buffer, pH 5·0, and 1·5 ml water or crude extract from Pectinol R-10 (850 μg protein). (○----○), Control; (●——●), with pectate lyase.

sintered glass to remove finer material.The filtrate was brought to pH 5·2 with citric acid and dialysed against water for 10 hr at 2°. The dialysing tube was changed every 2 hr, since Pectinol contains cellutotic enzymes.[4] The time-course of decomposition of pectins as followed turbidimetrically is shown in Fig. 1. The initial rise in turbidity is apparently due to the presence of pectin methyl esterase in either the Pectinol or the peach preparation, as pectic acid gives higher turbidity readings with cetyl pyridinium bromide (CPB) than the partially esterified compounds.[5] The initial rise in turbidity could be eliminated by first exposing the solution of commercial pectin to alkaline hydrolysis at pH 10·5.

[4] P. ALBERSHEIM, *Method. Enzymol.* **8**, 628 (1966).
[5] G. AVIGAD and Y. MILNER, *Israel J. Chem.* **5**, 175 (1967).

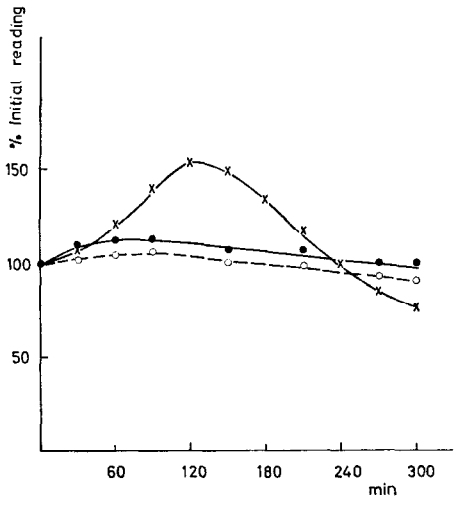

FIG. 2. PHENOL OXIDASES ACTIVITY AND DECOMPOSITION OF PECTIC SUBSTANCES ON INCUBATION OF THE SOLUBLE FRACTION FROM PEACHES WITH PARTIALLY PURIFIED PECTATE LYASE.

The reaction mixture consisted of 340 mg freeze-dried material from peaches in 17 ml 0·1 M phosphate–citrate buffer pH 5·0, and 600 μg pectate lyase in 3 ml water. Incubation was at 20°. Samples were withdrawn at intervals for turbidimetric determination of pectin and for polarographic measurement of activity towards 4-methylcatechol and quinol at pH 5·1 and 6·0 respectively. ×, Pectin; ○, activity towards 4-methylcatechol; ●, activity towards quinol.

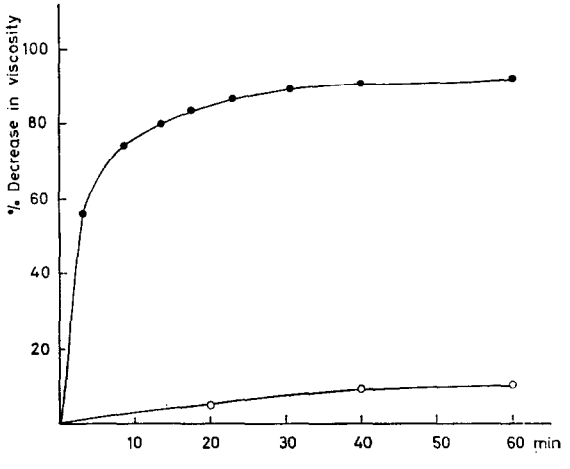

FIG. 3. THE EFFECT OF PECTATE LYASE ON THE VISCOSITY OF THE SOLUBLE FRACTION FROM PEACHES.

The reaction mixture consisted of 120 mg freeze-dried material from peaches in 6 ml 0·1 M phosphate–citrate buffer, pH 5·0, and 0·05 ml purified pectate lyase (1 μg protein) or water. ○, Control; ●, with pectate lyase.

Exposure of the soluble fraction from peaches to an unpurified extract of Pectinol resulted in a considerable loss of phenol oxidase activity. There was no difference in the relative loss of activities towards quinol or 4-methylcatechol. Here again, an initial rise in turbidity was observed, but was absent after alkaline hydrolysis at pH 10·5. It seemed probable that the loss in activity of phenol oxidases on incubation with pectinol is due to the presence of proteolytic enzymes, which are known to be present in this crude commercial preparation.

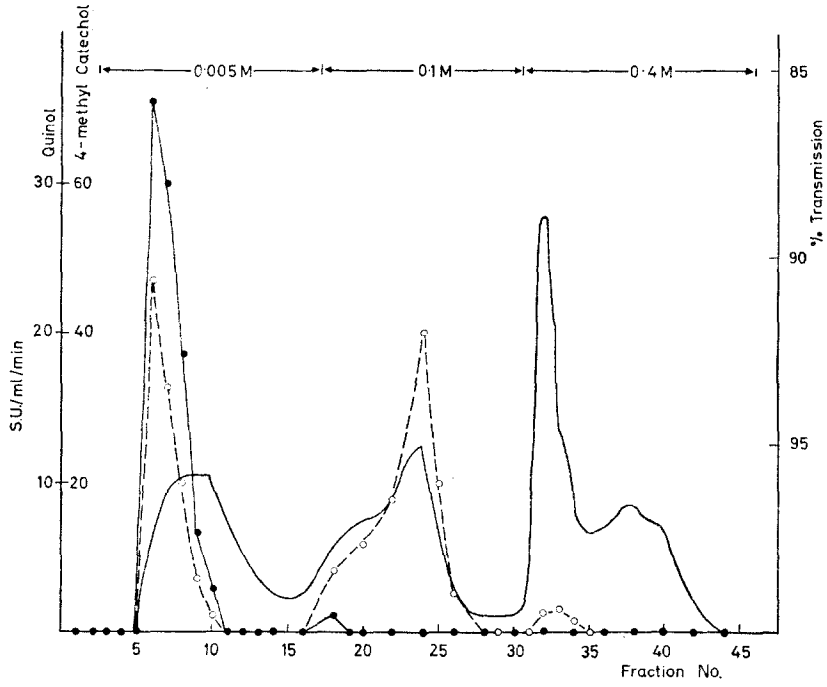

FIG. 4. ELUTION OF PHENOL OXIDASES FROM PEACHES ON A DEAE-CELLULOSE COLUMN, AFTER DECOMPOSITION OF THE PECTIC SUBSTANCES WITH PECTATE LYASE.

1 ml of purified pectate lyase (20 μg protein) was added to 380 mg freeze-dried material from peaches in 19 ml 0·005 M phosphate–citrate buffer, pH 5·0. The reaction mixture was incubated for 30 min at 25°. The pH was brought to pH 8·0 with NaOH and the solution passed through a DEAE-cellulose column (2·6 cm dia., 10 cm height) equilibrated with 0·005 M Na phosphate buffer, pH 8·0, at room temp. A step-wise elution was performed: 120 ml of 0·005 M, 0·1 M and 0·4 M phosphate buffer, pH 8·0. 8-ml fractions were collected at a flow rate of 1·5 ml/min. (●——●), Activity towards quinol; (○----○), activity towards 4-methylcatechol; (————), protein.

It was decided, therefore, to try and purify the pectate lyase before using it for the removal of pectins from the peach preparation. The procedure employed was basically that described by Albersheim.[4] A 32-fold purification was obtained. When the soluble fraction from peaches was incubated for 5 hr at 26° with this partially purified pectate lyase, only a small loss in the activities towards either phenolic substrate was observed (Fig. 2). Decomposition of the pectic material was slow and incomplete, when followed by the turbidity assay. A rapid decrease in the viscosity of the reaction mixture was, however, noted within a few minutes from the addition of pectate lyase. The changes in viscosity of the preparation from peaches

on addition of pectate lyase were followed using an Ostwald viscosimeter. A 90% decrease in viscosity within 30 min was observed (Fig. 3) when the preparation from peaches was incubated at 29° with a pectate lyase concentration 100-fold smaller than that used in the CPB assay. No loss of activity towards 4-methylcatechol or quinol occurred after 1 hr incubation under these conditions. No precipitation of pectins could be observed after this period of incubation on addition of 1·5 vol. of acetone or on 70% saturation with ammonium sulphate.

A preparation from peaches was treated with partially purified pectate lyase for 30 min at 25°. During this period, a 95% decrease in the initial viscosity of the preparation was observed while there was no loss in the activity towards either 4-methylcatechol or quinol. The hydrolysate was passed through a DEAE-cellulose column and a stepwise elution with

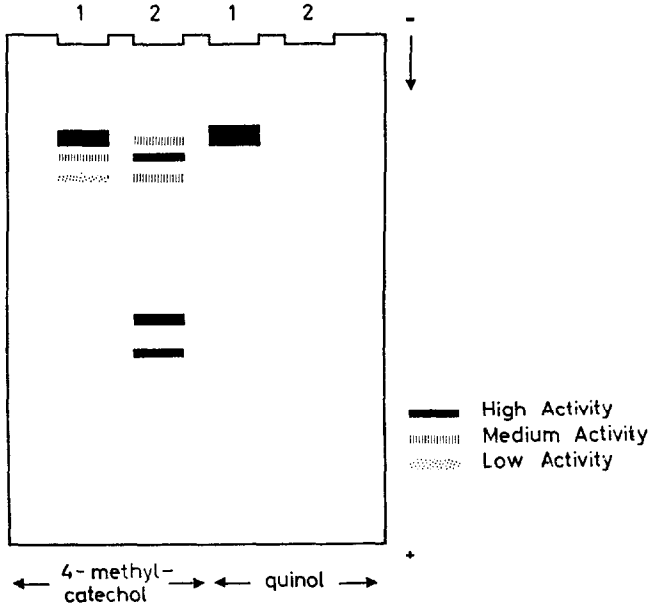

FIG. 5. ELECTROPHORESIS OF PHENOL OXIDASES FROM PEACHES IN ACRYLAMIDE GEL.

6% Gel in 0·02 M tricine buffer, pH 7·9. 270 V, 78 mA, 130 min run. (1) Fraction eluted from DEAE-cellulose at 0·005 M phosphate buffer, pH 8·0. (2) Fraction eluted from DEAE-cellulose at 0·1 M phosphate buffer, pH 8·0.

phosphate buffer, pH 7·3, was performed (Fig. 4). The activity towards 4-methylcatechol was separated into two fractions. One, which was not adsorbed by the column, is capable of oxidizing quinol. The rate of quinol oxidation by this fraction was 93% of the rate of oxidation of 4-methylcatechol, as compared to 25% before elution. Purification, calculated for the activity towards quinol, was 9-fold. The second fraction, eluted at 0·1 M phosphate buffer, pH 8·0, was inactive towards quinol. The overall recovery of the activity towards the two substrates was 91% for quinol and 73% for 4-methylcatechol.

The two fractions from DEAE-cellulose column were run electrophoretically in acrylamide gel. The gels were developed for enzyme activity with either 4-methylcatechol or quinol (Fig. 5). Activity towards quinol was restricted to one band and was present only in the fraction not adsorbed by the DEAE-cellulose column. This band was also active towards 4-methylcatechol and *p*-phenylenediamine. The fraction eluted from DEAE-cellulose with

0·1 M phosphate buffer, pH 8·0, was resolved into several bands capable of oxidizing 4-methylcatechol. No resolution was obtained with peach preparations which were not treated with pectate lyase. The preparation purified on DEAE-cellulose, which was active towards quinol, is quite unstable and there was a rapid loss of activity on its storage at 0–2°.

It appears that the use of purified pectate lyase offers a simple and effective method for the removal of pectic substances from fruit homogenates, before the isolation of plant enzymes. Pectate lyase has the advantage of being commercially available and its mode of action ensures a rapid reduction in the viscosity of fruit homogenates. The products of pectin breakdown can then be separated from proteins by ammonium sulphate or acetone fractionation or by gel filtration. It should be noted that when pectin decomposition is followed turbidimetrically its apparent rate of breakdown appears to be much slower than when followed viscosimetrically. This could probably be accounted for by the fact that viscosity decreases rapidly when pectin chains are cleaved at random. In the turbidimetric method, the reaction product fails to react with the quarternary ammonium compound only when the chain length is considerably reduced. Reid[6] calculated that the cleavage of only 0·5–1·0% of the internal glucosidic bonds of pectic substances would reduce the viscosity of their solution by 50%. Exopolygalacturonase, on the other hand, will have to hydrolyse 45% of the bands to achieve a 50% decrease in viscosity.[7]

EXPERIMENTAL

Peaches, variety "Salvey", were purchased at a local supermarket and stored at 2°. Destoned peaches were homogenized in 0·4 M sucrose 0·1 M phosphate buffer pH 7·3 containing 0·01 M sodium ascorbate and 10% Polyclar AT. The homogenate was filtered through gauze, centrifuged at 20,000 *g* for 30 min and to the supernatant, 1·5 vol. of cold (−15°) acetone were added. The precipitate was collected, freeze-dried and stored at −15°. Samples of the stored material were used throughout the experiments.

Catechol oxidase and laccase activities were determined by the use of a polarographic oxygen electrode as previously described.[8,9] The oxidation of quinol at pH 6·0 served as a measure for laccase activity. 4-Methylcatechol is oxidized by both catechol oxidase and laccase but the pH optima for the two enzymes are 5·1 and 6·0 respectively.[1,2] Pectate lyase activity was determined according to Albersheim.[4]

The decomposition of pectic substances in preparations from peaches by pectic lyase was followed by turbidimetric and viscosimetric methods. The turbidimetric method was basically that described by Avigad and Milner.[5] Viscosity was determined using a 5 ml Ostwald capillary viscosimeter at 29° in a constant temperature bath.

DEAE-cellulose was prepared for use according to Peterson and Sober.[10] The columns were coated with dimethyl-dichlorosilane before packing.

Acrylamide gel electrophoresis was carried out using the E-C Apparatus Corporation standard vertical gel cell equipped with a buffer pump. The gel was prepared by dissolving 11·4 g acrylamide, 0·6 g *N,N'*-methy lene bisacrylamide, 240 mg ammonium persulphate and 0·15 ml *N,N,N',N'*-tetramethyl-ethylenediamine in 200 ml 0·02 M tricine buffer (*N*-tris-(hydroxymethyl)-methylglycine) pH 7·9. The same buffer (0·02 M) was used in the electrode compartments. The samples were applied in buffer containing 20% sucrose and bromocresol purple, used as a marker. The runs were performed at room temp., using tap water for the cooling system. At the end of the run, the gels were dipped in 0·1 M phosphate–citrate buffer, pH 6·0, for 10–15 min and then developed for phenol oxidases activity by spraying with a 10 mM solution of the desired phenolic substrate containing 0·05% *p*-phenylenediamine in 0·1 M phosphate–citrate buffer, pH 6·0.

Protein was determined according to Lowry *et al.*[11]

Acknowledgements—This work has been supported in part by a grant FG-267 from the U.S. Department of Agriculture to A.M.M. Our thanks are due to Rohm & Haas for a sample of Pectinol R-10 and to General Aniline and Film Corporation for a sample of Polyclar AT. Our thanks are also due to Mr. E. Vaadja for technical assistance.

[6] W. W. REID, *Biochem. J.* **50**, 289 (1952).
[7] I. BARASH and E. ANGEL, *Israel J. Botany*, in press.
[8] E. HAREL, A. M. MAYER and Y. SHAIN, *Physiol. Plantarum* **17**, 921 (1964).
[9] A. M. MAYER, E. HAREL and R. BEN-SHAUL, *Phytochem.* **5**, 783 (1966).
[10] E. A. PETERSON and H. A. SOBER, *Method. Enzymol.* **5**, 3 (1962).
[11] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and J. RANDALL, *J. Biol. Chem.* **193**, 265 (1951).