[CONTRIBUTION FROM THE DEPARTMENT OF AGRICULTURAL BIOCHEMISTRY OF WEST VIRGINIA UNIVERSITY]

# The Enzyme Activities of Pectinol A on Pectin and Other Substances[1,2]

BY V. B. FISH AND R. B. DUSTMAN

Pectinol A and other pectic enzymes have been used for some years in researches on pectic material. Pectic enzymes are divided into three classes, protopectinase, pectinase and pectase. The purpose of this paper is to show some of the conditions affecting the pectinase and pectase activity of Pectinol A and to give evidence of its activity on several non-pectic substances.

It has been known for a long time that pectinase enzymes will bring about the hydrolysis of pectic substances to produce galacturonic acid and were first used by Ehrlich[3] for this purpose.

Quite recently Rietz and Maclay[4] reported the use of an enzyme preparation similar to Pectinol A for the production of galacturonic acid from pectin.

Kertesz[5] in 1936 reviewed the available information regarding pectic enzymes and gave the optimal *p*H for pectinase activity as 3.0 to 3.5. He also reported an optimal temperature of 30°.

Norman[6] has pointed out that it is difficult to evaluate accurately the pectinase activity of a preparation due to its complex nature.

Pectase activity has also been long recognized and a method was proposed in 1937 by Kertesz[7] for its estimation. Kertesz reported that pectinmethoxylase does not have an optimum *p*H since its action increases with decreasing hydrogen ion concentration until the effect of the alkalinity of the medium supersedes the enzyme activity. In his method a solution of pectin is adjusted to *p*H 6.2 with 0.1 *N* NaOH using methyl red indicator. Alkali is added, at frequent intervals of time, just sufficient to discharge all pink color of the indicator. In case the enzyme preparation imparted a color which would mask that of the indicator he suggested the use of electrometric titration.

## Experimental

**The Pectinase Activity of Pectinol A on Pectinum N. F. VII.**—The sample of Pectinum N. F. VII[8] used in most of the experiments described in this paper contained: uronic acid 82.3% as determined from the carbon dioxide produced by the action of 12.5% hydrochloric acid, moisture 8.65%, methoxyl 9.75% and ash 0.53%.

Since it was pointed out by Norman[6] that it is very difficult to measure pectinase activity quantitatively, no attempt was made to make such a determination. However, the pectinase action of Pectinol A was demonstrated by two independent methods. The changes in the optical rotation and reducing values of pectin solutions were measured during incubation with the enzyme. In order to conduct these experiments satisfactorily it was found necessary to remove the sugars from Pectinol A. The enzyme preparation was extracted for twenty-four hours with cold 80% ethanol in an apparatus which allowed freshly distilled alcohol to percolate through the sample and which siphoned off the dissolved material at intervals of about twenty minutes. At the termination of the twenty-four-hour period the insoluble matter was washed into centrifuge tubes with 80% alcohol and washed twice by centrifugation with 80% alcohol, once with 95% and twice with absolute alcohol. The material was finally washed twice with anhydrous ether and dried in warm circulating air. From 30.0 g. of Pectinol A, 2.28 g. of sugar-free enzyme was obtained; yield 7.6%.

An analysis of the extracted sugars showed that Pectinol A contained 62.3% dextrose, 24.3% levulose and 4.9% sucrose or a total of 91.5% sugars.

Two solutions containing 1 g. each of pectin were prepared and 10 ml. of a 1% sugar-free Pectinol A solution added to one of the pectin solutions. Another 10-ml. portion of the enzyme solution was boiled to inactivate the enzymes and rinsed into the second pectin solution. The solutions were diluted to 100 ml. with distilled water, mixed, and their optical rotation was measured at 25° in a 100-mm. tube. The initial reading for both solutions was +2.10°. The solutions were incubated at 37.5° and polarimeter readings were made on portions of each solution after it had been cooled to 25°, after 4, 10, 15, 20, 24, and 30 hours of incubation time. The readings obtained on the solution containing the active enzyme were +1.30, +0.75, +0.59, +0.48, +0.47, and +0.47°, respectively. The reading of the blank remained at +2.10° for the thirty-hour period.

If the optical activity of the various constituents of pectin other than galacturonic acid is disregarded, the final rotation after complete hydrolysis may be calculated. The pectin used was found to contain 82.3% uronic acid and the calculated rotation of a solution of 0.823 g. of galacturonic acid in 100 ml. of solution is +0.455°. On the basis of these data it seems that the hydrolysis of the polyuronide portion of the pectin was almost complete in twenty-four hours. The reaction of the mixture changed from *p*H 3.6 at the start of the experiment to *p*H 2.8 after twenty-four hours. This *p*H range was not greatly different from the optimal range reported in the literature for pectinase activity.[6]

Since pectinase activity is primarily associated with the hydrolysis of the polyuronide portion of pectic substances it is evident that the determination of increased reducing power of the hydrolysis mixture should serve as a relative measure of such activity.

A solution was prepared containing 1% pectin and 0.1% sugar-free Pectinol A. A similar solution was prepared containing inactivated enzyme to serve as a blank. The *p*H of the initial solution was 3.6. The initial reducing value was determined in each and the reducing values were determined at frequent intervals during incubation at 40° for twenty-eight hours. The reducing value of the blank remained constant throughout the experiment. For convenience these values are expressed as mg. of galacturonic acid per 100 ml. of solution. The iodine reduction method of Goebel[9] was used in this experiment.

(1) Published with the approval of the Director of the West Virginia Agricultural Experiment Station as Scientific Paper No. 336.

(2) Pectinol A is a commercial enzyme preparation designed for the clarification of apple juice and apple wine. It is represented as containing sufficient pectinase to hydrolyze the pectin contained in such products, but no claim is made that it is a pure pectinase. The preparation was supplied to us by its manufacturer, Rohm and Haas Company of Philadelphia.

(3) F. Ehrlich, *Biochem. Z.*, **251**, 216 (1932).

(4) E. Rietz and W. D. Maclay, THIS JOURNAL, **65**, 1242 (1943).

(5) Z. I. Kertesz, *Ergebnisse der Enzymforschung*, **5**, 233 (1936).

(6) A. G. Norman, "Biochemistry of Cellulose, Polyuronides, Lignin, etc.," Oxford University Press, London, 1937, p. 107.

(7) Z. I. Kertesz, *J. Biol. Chem.*, **121**, 589 (1937).

(8) Pectinum N. F. VII (pure citrus pectin, product No. 444) was supplied by the California Fruit Growers Exchange, Ontario, California. This material is hereinafter designated as pectin.

(9) W. F. Goebel, *J. Biol. Chem.*, **72**, 801 (1927).

TABLE I

EFFECT OF ONE HUNDRED MILLIGRAMS OF SUGAR-FREE PECTINOL A UPON THE OPTICAL ACTIVITY OF ONE GRAM OF SUBSTRATE IN ONE HUNDRED MILLILITERS OF SOLUTION WHEN INCUBATED AT 37.5° FOR VARIOUS PERIODS

| | Sucrose | | Inulin | | Starch | | Maltose | |
|---|---|---|---|---|---|---|---|---|
| Time, hr. | Obs. | Calcd. spec. | Obs. | Calcd. spec. | Obs. | Calcd. spec. | Obs. | Calcd. spec. |
| 0 | 1.35° | 67.5° | −0.69° | −34.5° | 3.54° | 177° | 2.62° | 131.0° |
| 4 | 0.54 | 27.0 | − .71 | −35.5 | 2.06 | 103 | 1.81 | 90.5 |
| 10 | − .02 | − 1.0 | − .74 | −37.0 | 1.56 | 78 | 1.40 | 70.0 |
| 15 | − .24 | −12.0 | − .75 | −37.5 | 1.42 | 71 | 1.30 | 65.0 |
| 20 | − .35 | −17.5 | − .80 | −40.0 | 1.38 | 69 | 1.28 | 64.0 |
| 24 | − .38 | −19.0 | − .80 | −40.0 | 1.36 | 68 | 1.28 | 64.0 |
| 30 | − .40 | −20.0 | − .80 | −40.0 | .. | ... | .. | ... |
| Calcd. final rotn. at complete hydrolysis | | −0.418 | | −2.05 | | 1.17 | | 1.06 |

The following values were obtained: blank, 145.0; after 2 hours 362.8; 4 hours, 527.7; 7 hours, 694; 10 hours, 791; 15 hours, 848; 18 hours, 853; 24 hours, 860; 28 hours, 864. In this experiment as in the one previously described little change was produced after twenty-four hours. In the first two-hour period 100 mg. of the enzyme preparation liberated aldehyde groups equivalent to 108.4 mg. of uronic acid per hour. Although the *p*H of the reaction mixture varied from 3.6 to 2.8 in twenty-four hours it became only slightly more acid than the lower limit of range reported in the literature as optimum for pectinase activity.[6]

**The Pectase (Pectin–methoxylase) Activity of Pectinol A.**—Since pectinase preparations have been found to show pectase activity,[5] it seemed advisable to investigate the pectase activity of Pectinol A and the sugar-free preparations made from it.

It was found that there was a considerable increase in titratable acidity produced when Pectinol A was allowed to act on pectin solutions as acid as *p*H 3.6 and that the activity continued even though the solutions became more acidic. It seemed rather strange that the demethoxylation should proceed in such acid solutions in view of the fact that Kertesz[5,7] found no optimal *p*H for pectase activity as its activity increased with increasing *p*H until the alkalinity of the medium caused extensive demethoxylation of pectin. The procedure proposed by Kertesz[7] was therefore modified in such a way as to allow the determination of pectin–methoxylase activity of enzyme preparations in solutions at a *p*H other than 6.2.

The method used in this work was as follows: Buffer solutions were prepared of *p*H 3, 4, 5, 6, 7 and 8 and were used to standardize a Fisher Senior Model Titrimeter over that range and at a temperature of 31°. Exactly 25 ml. of a 1% solution of pectin was placed in a 150-ml. beaker and about 20 ml. of water added. The solution was warmed to 31° in a water-bath heated by an adjustable electric hot-plate furnished with the instrument. When the solution was warmed to 31° it was adjusted nearly to the desired *p*H, which, when greater than 3.6, required the addition of alkali. Since the pectase activity of Pectinol A is relatively low, 0.02 *N* NaOH was used rather than 0.1 *N* as suggested by Kertesz. An accurately weighed portion of the enzyme preparation was then added, the time noted, and the solution adjusted to the desired *p*H. The volume of alkali thus added was recorded and the *p*H was maintained constant by the frequent addition of alkali. This procedure was continued for exactly one hour. The reaction mixture was then titrated to *p*H 7.0 and the total volume of 0.02 *N* NaOH added was noted. A 25-ml. portion of the same pectin solution was then titrated (at 31°) to nearly *p*H 7.0, the same weight of enzyme preparation added as was used in the preceding experiment, and then the titration was completed to *p*H 7.0. The volume of alkali so used served as a *blank* and the difference represented the acid produced by the demethoxylation of the pectin. Activities were calculated to the basis proposed by Kertesz,[7] that is, mg. of $CH_3O$ hydrolyzed by 1 g. of enzyme in thirty minutes. The reaction mixtures were finally titrated to *p*H 7.0 since from titration curves of both pectin and galacturonic acid *p*H 7.0 falls approximately at the point of inflection of such curves. In the determination of pectase activity in solutions more acid than the original pectin solution the *p*H was adjusted by the use of 0.02 *N* HCl and an equal amount of acid was added to the blank.

Using this method the pectin–methoxylase activity of a sugar-free sample of Pectinol A was determined at *p*H 3.0, 3.4, 3.8, 4.2, 4.4, 4.6, 5.0, 5.4, 5.8, 6.2 and 7.0. The activities found were 20.0, 29.0, 38.0, 45.3, 47.0, 46.5, 40.0, 30.0, 19.5, 10.5 and 4.0, respectively. The optimal *p*H found in these experiments for the pectin–methoxylase activity of this sugar-free sample of Pectinol A is therefore from 4.3 to 4.6. The pectase activity of another sugar-free sample of Pectinol A at *p*H 4.5 was 30.6, of Pectinol 100-D[10] was 38.7 and of Pectinol A (as received) was 4.2.

**The Action of Sugar-free Pectinol A on Some Non-pectic Substances.**—Two solutions each, of sucrose, inulin, soluble starch and maltose, were prepared with each solution containing 1.0 g. of substance dissolved in distilled water. Two solutions of sugar-free Pectinol A were prepared, one of which was boiled to inactivate the enzyme. The inactivated enzyme preparation was used with one solution each of the substrates to serve as a blank. All substrates were adjusted to *p*H 3.3 with acetic acid and an amount of enzyme solution containing 0.1 g. of enzyme preparation was added to each substrate. The solutions were then diluted to 100 ml. with distilled water. The optical rotation of the blanks was measured and all solutions were placed in an incubator at 37.5°. The readings were made at 25° in a 200-mm. tube. The optical rotation which should be attained at complete hydrolysis was calculated for each substrate and is included with other data obtained in Table I.

The calculated per cent. of hydrolysis for each of the substances listed in Table I at the time of the last reading is for sucrose 99.2, inulin 8.1, starch 91.9 and maltose 85.7. A similar experiment with a xylan suspension gave no indication of hydrolysis.

## Summary

Some of the properties of a commercial pectic enzyme preparation, Pectinol A, have been investigated.

Pectinol A was found to contain about 91.5% sugar, dextrose and levulose being the principal sugars present. It was shown that these sugars may be extracted with cold 80% ethanol, leaving an active sugar-free enzyme mixture.

It was shown by optical rotation and by iodine reduction methods that the sugar-free Pectinol A would completely hydrolyze ten times its weight of pectin to galacturonic acid in about twenty-

(10) Received from Rohm and Haas Company after most of the work herein reported was completed.

four hours at a temperature of from 37.5 to 40° while the *p*H of the reaction mixture changed from 3.6 to 2.8.

The pectase activity of Pectinol A, Pectinol 100 D and two samples of sugar-free Pectinol A were found to be as follows: Pectinol A, 4.2; Pectinol 100D, 38.7; sugar-free Pectinol $A_1$, 47.0, and sugar-free Pectinol $A_2$, 30.6, all measured at 31° and *p*H 4.5. These activities were determined by a modification of the method of Kertesz and are expressed as mg. of $CH_3O$ per thirty minutes per gram of enzyme.

The optimal *p*H range for the pectase activity of these samples of Pectinol A was found to be from 4.3 to 4.6.

It was shown by optical rotation methods that sugar-free Pectinol A will bring about the hydrolysis of sucrose, starch and maltose at 37.5° and *p*H 3.3. This enzyme showed only slight hydrolysis of inulin and gave no indication of hydrolysis of a suspension of xylan under these conditions.

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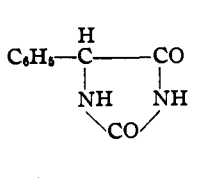
[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT OF THE UNIVERSITY OF COLORADO]

# The Glyoxalines. IV. A Study of the Structure of Certain Glyoxalines

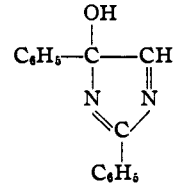
BY DAVID LLOYD WILLIAMS,[1] FRED L. SYMONDS,[2] JOHN B. EKELEY[3] AND ANTHONY R. RONZIO[4]

It has been shown that phenylglyoxal reacts with amidines, urea and thiourea to yield glyoxalines,[5,6] hydantoins[7] and thiohydantoins,[8] respectively.

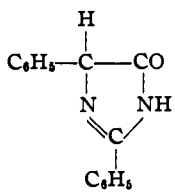
Urea was shown to yield 4-phenyl-hydantoin (I),[7] a compound which had been prepared previously by a method which left no doubt regarding the structure. Benzamidine was shown to yield a compound which could be formulated as either a 2,4-diphenyl-4-hydroxyglyoxaline (II), or as a 2,4-diphenyl-5-keto-dihydroglyoxaline (III).



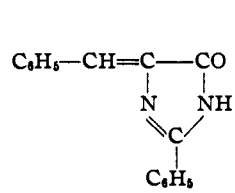
I



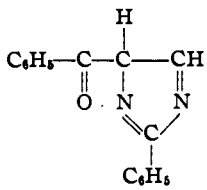
II



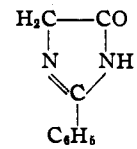
III



IV



V

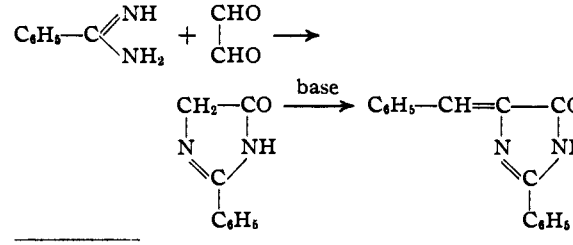


VI

Benzamidine, glyoxal and benzaldehyde react[9] in basic solution to give a compound which was shown by absorption spectra[10] to be related to benzal-phenyl-glyoxalidone (IV), first synthesized by Ruhemann and Cunnington.[11] The structure of a benzoyl-phenyl-glyoxalidone (V) was given to the compound.

The mechanism assumed for the reaction was as follows. The reaction of benzamidine and glyoxal was shown to yield an unstable addition product which easily decomposed in water solution to regenerate benzamidine and glyoxal.[9] In basic solution, the glyoxal was assumed to react with benzaldehyde to yield $C_6H_5COCHOHCHO$, which, in turn, was assumed to condense with benzamidine to yield compound (V).

However, should the reaction between benzamidine and glyoxal follow a path analogous to that of phenylglyoxal and urea, the product would then be 2-phenyl-5-keto-dihydroglyoxaline (VI). As is well known, the hydrogen atoms on position 4 in this class of compounds are reactive. Compound (VI) could then condense with benzaldehyde to yield a compound identical with Ruhemann and Cunnington's benzal-phenyl-glyoxalidone (IV), according to the equation



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(5) Waugh, Ekeley and Ronzio, THIS JOURNAL, **64**, 2028 (1942).

(6) Third paper of this series, Cole and Ronzio, *ibid.*, **66**, 1584 (1944).

(7) Fisher, Ekeley and Ronzio, *ibid.*, **64**, 1434 (1942).

(8) Unpublished work.

(9) Ekeley and Ronzio, THIS JOURNAL, **57**, 1353 (1935). See also Ekeley and Elliott, *ibid.*, **58**, 163 (1936).

(10) Ekeley and Ronzio, *ibid.*, **59**, 1118 (1937).

(11) Ruhemann and Cunnington, *J. Chem. Soc.*, **75**, 954 (1899).