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PROPERTIES OF TOMATO PECTASE*

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Pectase, the enzyme catalyzing the deesterification of pectin, has been used as a catalyst in the preparation of low ester pectins (1–3), partially deesterified products showing promise of commercial importance.

The literature on pectase prior to 1936 has been reviewed by Kertesz (4) who points out that many of these early studies are of doubtful value because the data were based upon gel formation, which is not a true measure of the reaction involved. The direct titration of liberated carboxyl groups is a valid measure of pectase action and has been used in recent studies on pectases derived from tobacco (4), tomatoes (5–8), alfalfa (9), and citrus peel (2, 10). These studies provide information regarding the general nature of the enzyme and indicate the non-identity of pectases derived from different sources.

The recent reports by Lineweaver and Ballou (9, 11) that pectase activity is influenced by cations has both theoretical and practical significance. It explains the earlier observation by Hills *et al.* (1) that tomato pectase activity was increased by the addition of sodium oxalate. The application of these findings to the measurement and utilization of pectase is obvious.

Only a few published studies are available regarding the properties of tomato pectase. Kertesz (5) studied the effects of substrate and enzyme concentrations on the determination of pectase activity. The optimum pH was not determined, but the data indicate that it is 6.2 or above. A subsequent study (6) showed that ripe tomatoes contain more pectase than immature fruits. Pectinase, the enzyme hydrolyzing the glycosidic linkages of the pectin molecule, was detected in ripe tomatoes but not in green tomatoes. Studies on the pasteurization of tomato juice (7) showed that tomato pectase was completely destroyed by heating to 80° for 45 seconds or to 70° for 2.5 minutes. The time required for complete inactivation was affected by the pH of the mixture, being less for juices acidified below the natural pH (about 4.0).

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The preparation of tomato pectase was studied by Willaman and Hills (8). They observed that the enzyme was adsorbed on the pulp solids at the natural pH of the tissue but was desorbed by the addition of alkali. Thus above pH 5.0, clear filtrates free of suspended solids and rich in pectase were readily prepared.

Tomato pectase was studied because it is a cheap and convenient catalyst for the deesterification of pectin. The present paper presents further data on the preparation and properties of tomato pectase.

Materials and Methods

Preparation of Pectase—The pectase solution used throughout this study was a clear yellow filtrate prepared from firm ripe tomatoes by the modification previously described (12) of the Willaman and Hills (8) method. Briefly, the method consisted in grinding firm ripe tomatoes to a pulp, adjusting to pH 7.5 by the addition of 5 N NaOH, and filtering. The extract was stored under xylene at 0°.

Preparation of Pectin—The purified apple pectin used as substrate in most of these studies was prepared from dried apple pomace by extraction with 14 parts by weight of water at 80° for 70 minutes at pH 2.5, oxalic acid being used as the acidulant. The extract was pressed out, treated with diastase to remove starch, filtered, and concentrated *in vacuo* to about 2 per cent pectin. The pectin was purified by the following modification of the method of Olsen *et al.* (13). 3 per cent of concentrated hydrochloric acid by volume was added to the pectin concentrate; the mixture was stirred and allowed to stand for 10 minutes, then precipitated by stirring into 2 volumes of 80 per cent alcohol. The precipitate was washed on a filter until free of chlorides, pressed, dried overnight at 60°, and ground to pass a 60 mesh screen. 4.9 per cent moisture, 0.39 per cent ash (moisture-free basis), and 10.24 per cent CH_3O (Zeisel), were obtained on the alcohol-free sample (12). The pectin was further characterized (14) as being 76.4 per cent methyl-esterified and containing 80.7 per cent methyl galacturonide.

Method of Pectase Determination—The method used to measure pectase activity was based upon the Kertesz (5) titration technique, in which the carboxyl groups liberated by pectase hydrolysis are titrated with standard alkali. The original method was modified by the use of a continuous electrometric titration at constant pH (1, 3) and by the addition of electrolyte as recommended by Lineweaver and Ballou (9, 11). The optimum conditions of pH and electrolyte concentration were determined and used in all measurements unless otherwise specified. This method is similar to the procedure used by Lineweaver and coworkers (9, 10).

The standard procedure was to measure into a 600 ml. beaker 200 ml.

of a 1 per cent solution of purified apple pectin, 12.5 ml. of 2 M sodium chloride, 5 ml. of 0.2 M sodium oxalate, and sufficient distilled water to give a final volume (after the addition of the enzyme) of 500 ml. The substrate thus contained 0.4 per cent pectin, 0.05 M sodium chloride, and 0.002 M sodium oxalate.

The reaction mixture was adjusted to 30° ± 0.1° and maintained at that temperature in a water bath. It was stirred by a small electrical stirrer, and the pH was measured by a Beckman pH meter equipped with extension leads. The enzyme solution (usually 5 ml.) was added and the mixture, while being stirred, was quickly titrated to pH 7.5 or slightly above. The amount of deesterification which occurred during the addition of pectase and the neutralization of the reaction mixture was usually less than 0.2 ml. of 0.5 N NaOH, and therefore could be ignored.

The pH meter was set at pH 7.50 (corrected for the pH drift caused by stirring (12)), and observations of time and alkali consumption were begun at the instant the galvanometer needle of the pH meter crossed the zero mark on the dial. This provided a very sensitive means for measuring the start and the end of the reaction. The pH was maintained constant within 0.1 pH unit by the dropwise addition of 0.5 N NaOH. Ordinarily the reaction was allowed to proceed until 3 or 4 ml. of 0.5 N NaOH had been consumed. By measuring the elapsed time to the closest second and applying a temperature correction (0.4 per cent per 0.1° deviation from 30.0°), the precision of the method, as determined by the average deviation of a number of duplicate determinations, was 0.02 ml.

For measurements made between pH 7.5 and 5.0, the titration values were corrected for the incomplete neutralization of the substrate. Below pH 5.0, it was necessary to use a method based upon the difference in titration (to pH 7.5) of two mixtures, one at constant pH for a given time and the other titrated immediately. This required a carefully standardized titration procedure, especially with respect to the time consumed in the final neutralization.

Discussion of Method—The choice of a method for enzyme assay and of units for expressing activity depends upon an understanding of the nature of the reaction involved. Published studies on pectase disagree as to whether the enzyme deesterification of pectin is a first order or a zero order reaction. Kertesz (4) reported that tobacco pectase followed a first order reaction, whereas Lineweaver and Ballou (9) found that the reactions catalyzed by alfalfa and pea vine pectases approximated a zero order reaction over the initial 50 per cent hydrolysis. More recently Speiser *et al.* (15) studied the kinetics of tomato pectase action on pectin but failed to prove whether the deesterification is a zero or first order reaction.

Fig. 1 shows that the course of tomato pectase deesterification is zero order for the initial 40 or 50 per cent hydrolysis but thereafter deviates from a zero order reaction. The deesterification does not correspond to a first order reaction over any portion of the curve. These conclusions

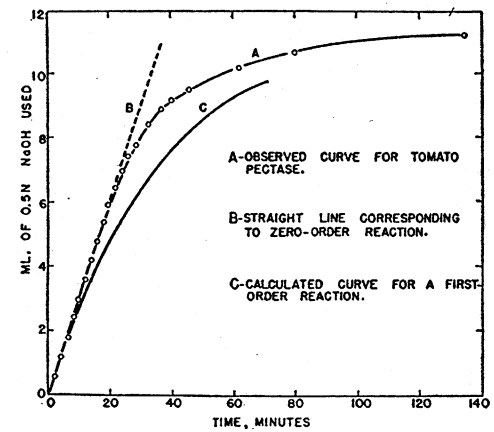

FIG. 1. Typical enzyme deesterification curve

TABLE I

Comparison of Zero and First Order Constants for Pectase Deesterification of Pectin (Standard Substrate Plus 5 Ml. of Pectase)

Fraction	Zero order, $K = \frac{x}{t}$ *	First order, $K = \frac{2.303}{t} \log_{10} \frac{a}{a - x}$
per cent		
0–10	0.150	0.0272
10–20	0.152	0.0317
20–30	0.150	0.0354
30–40	0.150	0.0414
40–50	0.140	0.0458
50–60	0.129	0.0512
60–70	0.099	0.0508
70–80	0.053	0.0377
80–90	0.021	0.0390
90–95	0.006	0.0205

* K is expressed as milliequivalents $\times$ min.$^{-1}$.

were confirmed by calculating the zero and first order constants for 10 per cent increments of the deesterification (Table I). From these data it is clear that the initial reaction is zero order instead of first order.

The present procedure, based upon the determination of pectase activity

within the region of the zero order reaction, satisfies the test for a valid method of measurement (16) in that the rate of activity is proportional to enzyme concentration over a wide range of added enzyme, as is shown later.

Pectase activity is expressed as the zero order velocity constant, where K is in units of milliequivalents of bonds hydrolyzed per minute. This unit is similar to that recommended by Lineweaver and Ballou (9). The ratio between the present unit and that proposed by Kertesz (5) is theorectically 1:930. However, since the Kertesz method does not measure pectase activity at the optimum pH or optimum electrolyte concentration, the ratio of the activities by the two methods may range from 1:300 to 1:800, depending upon the ion content of the pectin used as substrate.

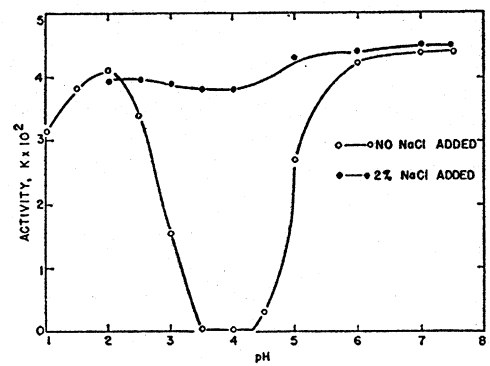

FIG. 2. Desorption of pectase from tomato pulp

Results

Desorption of Pectase from Tomato Pulp—The previously reported study of the desorption of pectase from tomato pulp by dilute alkali (8) has been extended to include the effects of acids and neutral electrolytes. Filtrates were prepared from 50 ml. portions of raw tomato pulp adjusted to the desired pH by the addition of either concentrated hydrochloric acid or 5 N NaOH. At pH 2.0 and below, the pectase activity of the filtrates was obtained by measurements made 30 and 60 minutes after the addition of acid, with extrapolation to zero time.

Fig. 2 shows that without added sodium chloride there is a region of minimum desorption near pH 4.0 with maxima at pH 2.0 and 7.5. The addition of 2 per cent sodium chloride was effective over the entire pH range investigated. It was also observed that at pH 6.0 or above the presence of 2 to 5 per cent of added sodium chloride caused a 10-fold

increase in the rate of filtration of the pectase extract through medium grade filter paper.

The use of 2 per cent sodium chloride at pH 6.0 or acidification to pH 2.0 without added salt would be suitable procedures for the large scale preparation of tomato pectase extracts. Extracts prepared at pH 2.0 should be adjusted to pH 4.0 prior to storage.

For the purpose of the present study, desorption at pH 7.5 without added salts was used in order to obtain an extract of low ionic concentration. The ion concentration of the extract was 0.11 M, determined by conductivity and calculated as KCl.

Stability of Tomato Pectase Extracts—In order to select the optimum conditions for storage and to anticipate the rate of deterioration, tomato pectase extracts were stored under various conditions of temperature and pH.

TABLE II

Effect of pH on Stability of Tomato Pectase Extracts Stored at 0° and 23°

pH	Rate of loss of activity	
	0°	23°
	per cent per mo.	*per cent per day*
7.5	2.6	1.42
5.0		0.55
4.0	1.0	0.42
3.0		0.88
2.0	12.7	20.0

50 ml. aliquots of tomato pectase extract were adjusted to the desired pH and stored at either 0° or at room temperature (23°). A few drops of xylene were added as a preservative. Pectase activity was determined at the start and at intervals during storage.

Table II shows the rate of deterioration of extracts stored at various pH values. The enzyme appears to be most stable at pH 4.0, the natural pH of tomato tissue. The addition of 2 per cent sodium chloride did not affect the stability of the enzyme at pH 4.0 and above but had a deleterious effect at a lower pH.

These data indicate that tomato pectase extracts may be stored for several days at room temperature or several months at 0° without appreciable loss. The pectase derived from tomatoes appears to be much more stable than that derived from citrus peel (10) or from alfalfa (9).

Effect of Enzyme Concentration—The activity per ml. of pectase extract, with the standard substrate, was observed to be constant over a wide range

of enzyme concentration (Table III). It was not possible to make accurate measurements with amounts of pectase extract greater than 20 ml.

The constancy of the pectase activity per ml. of added enzyme is important in the determination of pectase activity and appears to hold for all concentrations commonly encountered. It also indicates the validity of the method used for pectase determinations.

TABLE III

Effect of Enzyme Concentration on Pectase Activity

Tomato pectase added	Activity
ml.	*m.eq. per ml. per min.*
20.0	0.0285
10.0	0.0286
5.0	0.0287
2.0	0.0286
0.5	0.0284

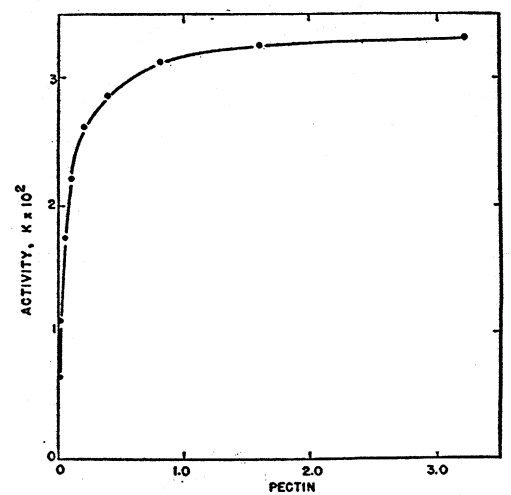

FIG. 3. Pectase activity as a function of substrate concentration

Effect of Substrate Concentration—The dependence of pectase activity on pectin concentration was determined by measuring the rate of deesterification of substrates containing amounts of pectin ranging from 0.01 to 3.2 per cent. In all cases, readings were taken before 40 per cent of the available methyl groups had been hydrolyzed.

The data (Fig. 3) indicate that pectase activity increases with pectin concentration, practically attaining a plateau above 1.6 per cent pectin.

At 0.4 per cent pectin, the concentration used for pectase assay, the activity was 85 per cent of that at 3.2 per cent pectin.

It was of interest, both from a practical and a theoretical standpoint, to determine the concentration of pectin which will give the maximum rate of deesterification per unit of enzyme. This was done by applying the Lineweaver and Burk (17) form of the Michaelis-Menten (18) equation

$$\frac{(S)}{v} = \frac{(S)}{V} + \frac{K_s}{V}$$

where (S) is the substrate concentration, v the observed velocity at a given substrate concentration, V the maximum velocity, and K_s the Michaelis-Menten constant. By plotting $(S)/v$ against (S), it was possible to determine V and K_s from the slope $1/V$ and the intercept K_s/V.

The maximum velocity was calculated to be 0.0334 (milliequivalent per ml. per minute), corresponding to about 3.2 per cent pectin concentration. The value for the Michaelis-Menten constant, 0.041 per cent, may be compared with the values 0.04 per cent for alfalfa pectase (9) and 0.08 per cent for citrus peel pectase (10).

It should be pointed out that the Michaelis-Menten equation applies only to the initial 40 per cent of the pectase deesterification curve. Beyond this point in the deesterification reaction there was a marked curvature in the plot of $(S)/v$ *versus* (S). This is not entirely unexpected because, as the deesterification reaction proceeds, the substrate changes both quantitatively (with respect to methyl ester content) and qualitatively (with respect to magnitude of the negative charge). The Lineweaver-Ballou hypothesis (9) concerning the formation of an inactive ionic complex between pectinic acid and pectase offers a possible explanation of this behavior.

Effect of Electrolyte Concentration—The recent studies by Lineweaver and Ballou (9) and by MacDonnell *et al.* (10) indicate the dependence of pectase action on electrolyte (cation) concentration. The concentration required for maximum pectase activity is a function of the pH and of the valency of the cation.

The fact that alfalfa and citrus peel pectases show differences in behavior toward cation concentrations indicates that this effect is also a function of the source of the enzyme.

In the present study the effect of sodium chloride concentration on tomato pectase activity was determined at pH 7.5, 6.0, 5.0, and 4.0. The results (Fig. 4) show that both the optimum salt concentration and the maximum activity are affected by pH. The optimum salt concentrations at pH 7.5, 6.0, 5.0, and 4.0 were 0.05, 0.08, 0.15, and 0.30 M Na ion, respectively. These values are nearly identical with those reported for alfalfa pectase (9) but are appreciably smaller than those for citrus peel pectase (10).

It was impossible to obtain enzyme-substrate mixtures entirely free of cations because small amounts were contributed by the alkali used for the original neutralization (1.5 milliequivalents), by the ash of the pectin substrate (0.5 milliequivalent), and by the pectase solution (0.5 milliequivalent). When dialyzed pectase was used without added electrolyte, the activities at pH 7.5 and 6.0 were 0.0054 and 0.00026 milliequivalent per ml. per minute, or 18 and 1 per cent of the respective maxima at optimum salt concentrations.

Tomato pectase activities at pH 7.5 in the presence of 0.05 M sodium acetate, sodium chloride, or potassium chloride were in the ratio of 100:97:97, indicating that the anion may have a slight effect and that sodium and potassium are equivalent.

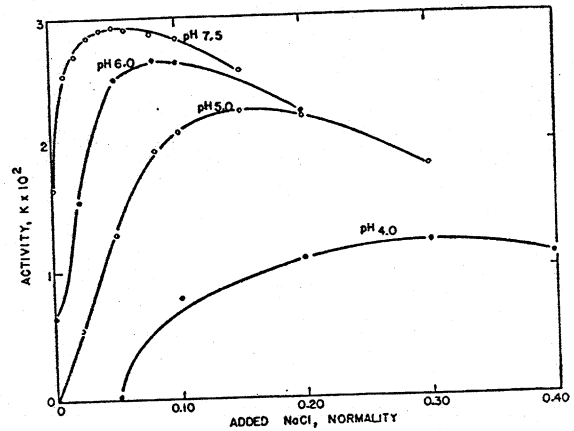

FIG. 4. Effect of NaCl on pectase activity at various pH levels

Effect of pH on Pectase Activity—The optimum pH for tomato pectase was determined in the presence of 0.05 M sodium chloride. Measurements between pH 7.5 and 5.0 were made by direct titration at constant pH, the results being corrected for incomplete neutralization of the substrate. At a lower pH, the results were obtained by titrating (to pH 7.5) two mixtures that had been allowed to react for different times, as described previously. Above pH 7.0, it was necessary to correct for the amount of deesterification caused by alkali. In Fig. 5, Curve A-B represents the observed rate of deesterification produced by enzyme and alkali together. Curve C shows the rate for alkali alone, and Curve A-A′ for the enzyme alone.

Tomato pectase in the presence of 0.05 M sodium chloride shows a maximum activity at pH 7.5 with a fairly broad optimum region extending from about pH 6.0 to 9.0. The data presented previously (Fig. 4) show that the optimum salt concentration increases at a lower pH. Thus 0.05 M NaCl, which is optimum for pH 7.5, is suboptimum for pH 6.0 or 5.0. From a

practical point of view it is desirable to show the maximum activity and required salt concentration in the range of pH below 7.5. These results, plotted in Curve D, Fig. 5, show that at a lower pH a material increase in pectase activity may be realized by increasing the salt concentration.

Temperature Coefficient—The influence of temperature on the rate of pectase action was studied under conditions nearly identical with those recommended for the large scale preparation of low ester pectins (19). Rate measurements were made at pH 6.5 and at temperatures ranging from 10–65° (Fig. 6). Readings were taken over a period of 30 minutes, although

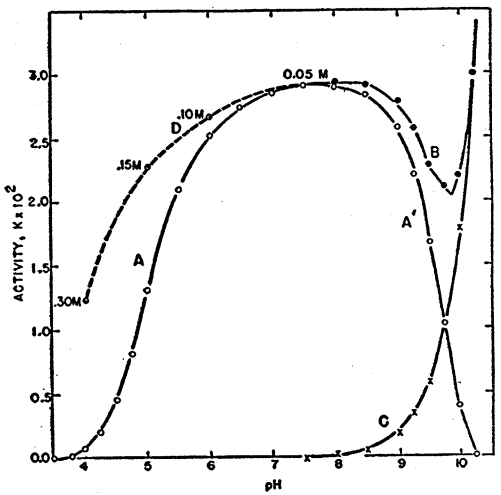

FIG. 5

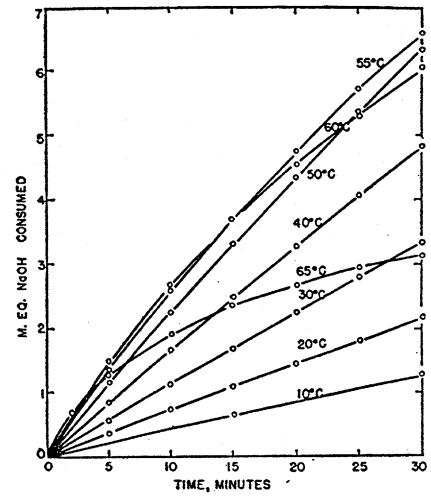

FIG. 6

FIG. 5. Activity-pH curve for tomato pectase. Curve A-B, observed deesterification; Curve C, deesterification by alkali; A-A′, corrected curve for pectase alone; Curve D, activity, pH 4.0 to 7.5, with optimum NaCl concentration for each pH.

FIG. 6. Effect of temperature on rate of pectase action at pH 6.5. Substrate, 0.8 per cent pectin, 0.05 N NaCl, 0.002 N Na oxalate. 2.0 ml. of tomato pectase extract used.

in some instances the reaction was allowed to continue for 1 hour. In all cases, the readings were confined to the initial 40 per cent portion of the deesterification reaction. Thus any deviation from linearity in these data is an indication of enzyme denaturation.

The "optimum temperature" for pectase action decreased with time, because at increased temperatures denaturation of the enzyme became an important factor. For a 2 minute reaction time the optimum temperature was 65°, for a 30 minute reaction it was 55° and for 1 hour (data not shown) it was 50°.

The linearity of the curves for 10°, 20°, 30°, and 40° indicates that denaturation of the enzyme was negligible under these conditions.

The increase in rate of an enzyme reaction with increase in temperature may be conveniently expressed as the Q_{10} value, the ratio of K_{t+10}/K_t. The Q_{10} values for tomato pectase shown in Table IV are much lower than the range of 2 to 3 predicted by the van't Hoff rule for catalyzed reactions in aqueous solution, and lower than is generally observed for other enzymes (20).

TABLE IV

Temperature Coefficient of Tomato Pectase Deesterification of Pectin

0.8 per cent pectin substrate, 0.05 M NaCl, 0.002 M Na oxalate, pH 6.5.

Temperature interval	Temperature coefficient, Q_{10}
°C.	
10–20	1.60
20–30	1.52
30–40	1.44
40–50	1.32

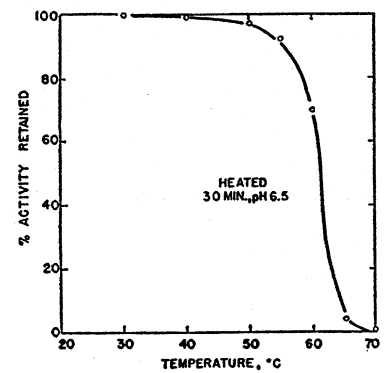

FIG. 7. Inactivation of pectase by heat

A value of 7280 calories for the activation energy was determined from the slope of $1/T$ *versus* log K (zero order). Initial reaction velocities were used in the calculations and the data were confined to the temperature range of 10–40° at which heat inactivation of the enzyme is not apparent. This is higher than the value 5790 calories previously reported by Speiser *et al.* (15), who used slightly different experimental conditions and based their results on the analysis of the isolated reaction product. A value of 6000 calories has been reported for citrus pectase (2).

Heat Inactivation—Fig. 7 shows the loss in activity caused by heating a

tomato pectase solution for 30 minutes at pH 6.5. The temperature (62°) required for 50 per cent inactivation is about 12° higher than that required for citrus peel pectase (10). This agrees with the general observation that tomato pectase is more stable in aqueous solution than is alfalfa or citrus peel pectase.

DISCUSSION

The differences in properties between tomato pectase and other plant pectases, while chiefly quantitative, are distinct enough to indicate the non-identity of pectases from different sources. The optimum pH and salt concentration for tomato pectase are pH 7.5 and 0.05 M NaCl. These values may be compared with pH 7.5 and 0.15 M NaCl for orange peel pectase (10) and pH 6.5 (salt concentration not determined) for tobacco pectase (4). Alfalfa pectase (10) is unique in that it shows maximum activity over the entire range from pH 5.6 (0.15 M NaCl) to pH 8.0 (0.02 M NaCl). Tomato pectase extracts are much more stable at 0° or room temperature than are extracts derived from alfalfa or citrus peel. Tomato and citrus pectases show differences in the effects of pH and salts on stability. The temperature required for heat inactivation of tomato pectase is about 12° higher than for citrus peel pectase.

On a fresh weight basis, the pectase activity of ripe tomatoes is about the same as that of fresh citrus peel and about 5 times that of fresh alfalfa (10). Crude pectase extracts prepared from tomatoes are about as active as the whole tissue. The efficient extraction of the enzyme from citrus peel or alfalfa requires the addition of several volumes of solvent, and the resulting extracts would be much less active than tomato extracts. Crude tomato pectase extracts may be prepared without added solvent and are sufficiently stable to permit their storage without further purification.

The present study indicates various means for improving the efficiency of pectase action. The activity per unit volume of pectase may be increased by deesterifying at a pH near 7.5 and adding the optimum quantity of electrolyte. The fact that pectase activity increases with increased substrate concentration may be applied to commercial practice by concentrating the pectin extract before deesterification. The use of increased temperatures is an obvious method of increasing the efficiency of pectase action. For most purposes, however, this method encounters the limitations imposed by the susceptibility of the pectin substrate to heat degradation (19). A fuller discussion of the utilization of tomato pectase for the large scale preparation of low ester pectins is presented in a separate publication (19).

SUMMARY

1. The deesterification of pectin by tomato pectase follows a zero order (for the initial 40 per cent) rather than a first order reaction. This fact

was utilized in determining reaction rates and in choosing units for expressing activity.

2. Tomato pectase was prepared from tomato pulp by alkaline desorption. The desorption may be accomplished also by acid or neutral electrolytes.

3. The relation of enzyme activity to substrate concentration conforms to the Michaelis-Menten theory. The K_s value of 0.046 per cent is comparable to that found for alfalfa pectase but differs from that for citrus pectase.

4. Tomato pectase has a broad optimum pH region, with a maximum at 7.5.

5. The addition of salts is necessary for maximum activity. The optimum salt concentration at pH 7.5 is 0.05 M NaCl but is greater at lower pH values.

6. The enzyme was most stable in aqueous solution at pH 4.0. At this pH and 0° it deteriorated about 1 per cent per month. A temperature of 70° was required for complete inactivation in 30 minutes at pH 6.5.

7. The temperature coefficient (Q_{10}) was 1.52 for the region of 20–30°. The Arrhenius energy of activation in the region from 10–40° was 7280 calories. Above 40° inactivation of the enzyme became apparent.

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