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Title THE ISOLATION AND CHARACTERIZATION OF THE PECTIC ENZYMES AND THE PECTIC SUBSTANCES OF THE NORTHWEST STRAWBERRY

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Enzymic systems able to hydrolyze the compounds forming the supporting structure of plant tissues have a major effect upon the texture of fruit products.

The objective of this thesis was the development of a theory to explain the textural changes in fresh and processed strawberries. The presence of pectinolytic enzymes and the substrates upon which they act were studied. In addition the activity of cellulase was investigated.

The following conclusions were made:

1. An endopolymethylgalacturonase exists in Northwest strawberries which catalyzes the hydrolysis of pectins, pectates and protopectins.

2. The strawberry endopolymethylgalacturonase indicated a maximum activity at pH values between 4.5 and 5.5.

3. The degree of methylation of the substrate did not appear to have an influence upon the activity of the enzymes. This enzyme demonstrated the same rate of action upon Na pectate and citrus pectin.

4. Sodium chloride solutions at concentrations up to 0.50 M and calcium ion at concentration 0.01 M did not show any effect on the activity of the strawberry endopolymethylgalacturonase in citrate buffer at pH 5.0.

5. The strawberry endopolymethylgalacturonase was inactivated after heating at 212°F for approximately 35 minutes in citrate buffer at pH 5.0.

6. Non-enzymic hydrolysis of the strawberry pectic substances occurs and hydrolysis is more pronounced at lower pH values.

7. The pectinesterase activity in Northwest strawberry is low in comparison with tomato fruits. The optimum activity of strawberry P.E. occurred at pH 7.5.

8. While cellulase activity existed in Northwest strawberries, the strawberry cellulase did not hydrolyze the insoluble strawberry cellulose.

THE ISOLATION AND CHARACTERIZATION OF THE PECTIC ENZYMES AND THE PECTIC SUBSTANCES OF THE NORTHWEST STRAWBERRY

by

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ISOLATION AND CHARACTERIZATION OF THE PECTIC ENZYMES AND THE PECTIC SUBSTANCES OF THE NORTHWEST STRAWBERRY

INTRODUCTION

Enzymic systems able to hydrolyze the compounds forming the supporting structure of plant tissues have a major effect upon the texture of fruit products.

Pectic enzymes influence the consistency of fruit products because of their ability to hydrolyze pectic substances. Such enzymes include the group of the polygalacturonases, the pectin transeliminase and the methylesterase. In addition to the pectic enzymes, the cellulases contribute to the softening of fruit products by attacking the cellulose which comprises the main supporting structure of plant tissues. The possibility exists that these enzymes are synthesized by the plant tissues or that they are formed by microorganisms living in or on the plant tissues.

The texture of fruit products is of prime importance because of its effect on consumer preference. Strawberries suffer a loss in consistency when they are stored fresh and during preservation by canning, freezing and freeze-dehydration. The cause of this texture deterioration in strawberries is not known. It is assumed that an enzymic or chemical hydrolysis of pectins and/or cellulose is the reaction mechanism.

In this study the presence of enzymes in the Northwest strawberry, which could catalyze the hydrolysis of pectic substances or cellulose, and the nature of the substrates of these enzymes were investigated.

REVIEW OF LITERATURE

Pectic Substances

Chemical Constitution and Nomenclature

Pectic substances are high molecular weight acid polysaccharides which are widespread in the plant kingdom (19, p. 341). These substances are polyuronides composed mostly of anhydrogalacturonic acid residues. The basic structure of these materials, the polygalacturonic acids, are thought to be composed entirely of anhydrogalacturonic acid residues (Figure 1) although other carbohydrates, for example arabinose, galactose, sorbose, rhamnose, could be attached to the chains of anhydrogalacturonic acid units (46, p. 5). Many a pectin preparation contains other sugar units

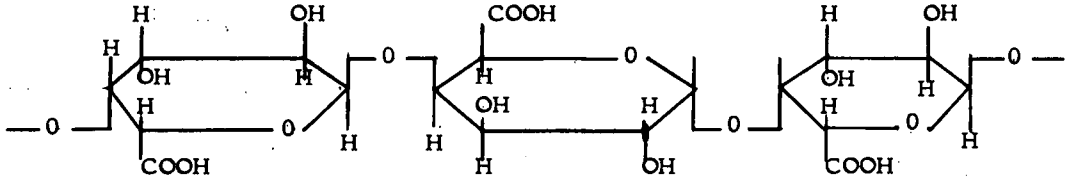


Figure 1. Polygalacturonic acid (pectic acid) chain.

along with galacturonic acid, probably in accompanying polysaccharide impurities or linked as side groups to the polygalacturonic acid chain (19, p. 344; 40, p. 47-52; 65). Enzymic analysis using

partially reduced pectins indicates that the main chain probably does not contain sugars other than anhydrogalacturonic acid. The molecular weight of pectins has been determined by viscometric measurements (40;51) and by sedimentation in the ultracentrifuge. Commercial pectin preparations usually have molecular weights between 10,000 and 20,000 (19, p. 344).

The carboxyl groups of pectin are partially esterified with methanol and in some cases the hydroxyl groups are partially acetylated (19, p. 344). By partial enzymic hydrolysis of pectin, oligogalacturonic acids are obtained. Mono- to pentamers have been identified by chromatography (66; 53; 29). The constitution of di- and trigalacturonic acids has been elucidated (38;39). The α-1,4-glycosidic type of linkage between the monomers in the pectin macromolecule has thus been confirmed. The pyranose ring of D-galacturonic acid probably occurs mainly in the chair 1 form (Figure 2) corresponding to the most stable conformation of

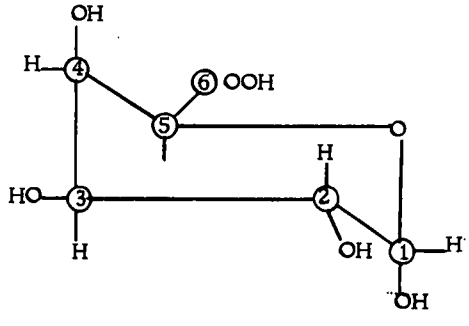


Figure 2. Galacturonic acid.

D-galactose (19). As both hydroxyl groups of D-galacturonic acid at the carbon atoms 1 and 4 are in axial position, the resulting polymer belongs to the trans-1, 4-polysaccharides (25). The free rotation at the glycosidic linkages is thereby hindered. Hence, the pectin macromolecule may be considered a chain with restricted flexibility. Since the secondary hydroxyl groups at the carbon atoms 2 and 3 and the carboxyl group at the carbon atom 5 are in equatorial position, they are easily accessible. The following nomenclature for pectic substances was adopted by the American Chemical Society in April 1944 (6).

"Pectic substances: Pectic substances is a group designation for those complex, colloidal carbohydrate derivatives which occur in, or are prepared from, plants and contain a large proportion of anhydrogalacturonic acid units which are thought to exist in a chain-like combination. The carboxyl groups of polygalacturonic acids may be partly esterified by methyl groups and partly or completely neutralized by one or more bases."

"Protopectin: The term protopectin is applied to the water-insoluble parent pectic substance which occurs in plants and which, upon restricted hydrolysis, yields pectinic acids."

"Pectinic acids: The term pectinic acids is used for colloidal polygalacturonic acids containing more than a negligible proportion

of methyl ester groups. Pectinic acids, under suitable conditions, are capable of forming gels with sugar and acid or, if suitably low in methoxyl content, with certain metallic ions. The salts of pectinic acids are either normal or acid pectinates."

"Pectin: The general term pectin (or pectins) designates those water-soluble pectinic acids of varying methyl ester content and degree of neutralization which are capable of forming gels with sugar and acid under suitable conditions."

"Pectic acid: The term pectic acid is applied to pectic substances mostly composed of colloidal polygalacturonic acids and essentially free from methyl ester groups. The salts of pectic acid are either normal or acid pectates."

Extraction of Pectic Substances from the Plant Tissue

The living tissue must be killed before extraction of cellular or cell-wall constituents and prepared so as to allow ready penetration of the pectin macromolecules. Enzyme activity must be avoided during disintegration of the tissue. Owens et al. (74) recommended the commonly used plant-tissue preparation procedure of slicing the tissue directly into hot 95 percent alcohol and heating to inactivate the pectic enzymes. They suggested that the temperature of the alcohol should be above 70°C, the final ethanol concentration should

be not less than 70 percent and the heating period should be about 12-18 minutes. Gee et al. (28) prepared marc by adding a sample of fresh plant tissue to three volumes of 95 percent alcohol and allowing the mixture to stand for at least one hour for enzyme inactivation, partial dehydration and extraction of the soluble solids from the pulp. Under these conditions inactivation of enzymes is limited largely to the surface (40, p. 18). Joslyn and Deuel (41) compared different methods of preparing alcohol-insoluble solids from apple tissue. Enzymic browning during preparation markedly decreased the solubility of pectins but did not alter the composition of pectins extracted. The solubility of pectins present in fresh or dried commercial apple pomace was markedly lower than that from carefully prepared nonoxidized apple tissue. Baker and Woodmansee (7) compared three polyphosphates--sodium hexametaphosphate, sodium tetraphosphate and tetrasodium pyrophosphate--in the extraction of pectin from apple pomace after boiling 20 minutes in hydrochloric acid solutions. Polyphosphates increased the yield of pectin at pH values above 3. McCready et al. (67) found that the effectiveness of polymetaphosphate and polyphosphate was nil at pH 2 and increased up to pH 3.5.

Sodium salts of ethylenediaminetetraacetic acid can be used as chelating agents at pH values above 6. Under these conditions the pectins in the plant tissue may be degraded (40, p. 37)

Extraction of pectin from apple pomace was complete with 0.10 N HCl by boiling for 30 minutes (90). The same authors reported that 0.5 percent ammonium oxalate at 100°C (30 minutes) gave a 94 percent yield of pectin.

Enzymes have been used to a limited extent in determining the nature of native pectins (40, p. 38).

Analysis and Characterization of Pectins

Crude alcohol precipitates of pectic substances contain mineral constituents, nitrogenous constituents, polysaccharides, tannins, flavonoids and other plant pigments. The total amount of these constituents may vary from 10 percent to 50 percent by weight of the pectins obtained (40, p. 48). Successive solution in water and reprecipitation with acidified alcohol has been used as a method of purification (26). Precipitation with acidified alcohol reduces the ash content, frees the pectin from other impurities and gives more complete recovery of pectins (40, p. 53). Dialysis against distilled water and ion exchange with cation exchange resins removed inorganic matter (50) but not the non-uronide organic material.

There is a variety of methods for determining acetyl, methoxyl and uronide content of the pectins. The methyl ester content of pectin preparations is usually determined by saponification (74) or by some modification of the Zeisel method (46, p. 231). Acetyl content can be determined by saponification and distillation of the acetic acid liberated (48) or by the ferric hydroxamate procedure (64). The uronide content of pectin preparations has been determined by direct titration (33), by decarboxylation (46, p. 36) by the carbazole method of Dische (22) or by the anthrone method (31). In the carbazole method, 5-formylpyroracemic acid is formed as an intermediate (98), while in anthrone test a furfural derivative is the intermediate. The color reaction with carbazole was more intense when the pectins were first de-esterified with alkali (63).

Viscosity of Pectic Solutions

The aqueous solutions of high molecular pectins are viscous. Viscosity is often a sensitive indication of differences or changes in colloidal solutions. The Ostwald capillary viscosity pipette is the instrument most commonly used for determining the relative viscosity of pectinic acid solutions (46, p. 163). The Ostwald-Cannon-Fenske pipette is also suitable. The viscosity is not governed by the methyl ester content but rather by the average molecular weight or size of

the pectinic acid. The viscosity of a solution increases with the concentration (46, p. 164).

pH has an effect upon the viscosity. Upon lowering the pH of pectinic acid solution by the addition of an acid, the viscosity decreases down to pH 2.2 to 2.4, after which there is no further change. Upon the addition of sodium hydroxide the viscosity of low-ash pectinic acid increases and reaches a maximum at around pH 6 (97). As the temperature is elevated the viscosity of the solution is reduced. Temperatures above 50°C caused degradation of the pectinic acid and reduced the viscosity irreversibly (46, p. 167). Electrolytes decrease the viscosity, if they do not coagulate the pectins (19, p. 354).

State of Pectins in Plant Tissues

In most of the fruits examined, the extent of pectin esterification increased at first and then decreased during maturation. The extent and rate of the decrease is greater in fruits, like pears, that contain active pectinesterases (37; 67). The extent of acetylation of fruit pectin apparently increases during maturation and in storage (67). On the assumption that pectins exist essentially as 1, 4-linked galacturonans, OH groups in positions 2 and 3 that are not acetylated and COOH groups that are not esterified with methanol

would be available for chemical linkages. These free OH groups and COOH groups may form a variety of compounds, either with neighboring polygalacturonide chains or with associated constituents such as cellulose, hemicellulose, protein and lignin. Such compound formation may be involved in protopectin (40, p. 5). Protopectin, which is insoluble, is found in close association with other cell-wall constituents (40, p. 5). Bonner (13, p. 99) stated that protopectin may be composed of large molecules which are insoluble and on partial degradation become noticeably soluble in water. This contention is supported by the fact that nitropectins of high molecular weight can be obtained by nitration of pectins, in situ.

The high molecular weight and size of the protopectin macromolecule may be due to secondary aggregation of the filamentous macromolecules of pectin with one another or with other high polymers of the cell wall. Pallmann et al. (40, p. 104) suggested that this secondary aggregation may be produced by mechanical interlacing of the filamentous macromolecules of pectin with each other or with other highly polymerized constituents of the cell wall (cellulose, hemicellulose, lignin).

Compound formation between pectins and cellulose was proposed very early to account for the difficulty in extracting pectins with mild reagents (40, p. 66). Two types of combinations have

been proposed: ester formation between the free carboxyl groups of pectin and the hydroxyl groups of cellulose, or connection between the free carboxyl groups of pectin and the carboxyl groups of cellulose (40, p. 66).

Another possibility is the ester formation or etherlike linkages between pectins and other sugars. Araban, galactan, galactose, arabinose were reported in moderate concentrations, and mannose, xylose and rhamnose were found in trace amounts in apple pectin prepared from commercial liquid pectin by precipitation with methanol at 66 percent concentration (103).

Neukom et al. (72) reported data on separating the polysaccharides of sugar beet by chromatographic fractionation on diethylaminoethyl-cellulose columns. The araban and galactan components could not be separated from polygalacturonide components by elution with buffers of increasing pH value. Such separation would be expected if they were present as mixtures, for they would be neutral in charge in comparison with the negative charged polygalacturonides.

Salt linkages between the carboxyls of pectins and basic groups of proteins have been considered to be a possible explanation for the insolubility of protopectin (40, p. 80). Painter (75) reported that proteins present in milled red alga interfered with the extraction

of polysaccharides by hot aqueous sodium acetate at pH 7. The possibility that proteins may contribute to the difficulty of extracting pectins from apple-tissue and apple pomace is supported by the fact that apple-tissue proteins are difficult to extract and appear to extract best under alkaline conditions which also remove protopectin (40, p. 82). The possibility was proposed that the formation of compounds between lignins and pectins may contribute to the insolubility of protopectins. Chemical combination between lignin and carbohydrates has been indicated by the demonstration of the existence of phenylglycosidic bonds in wheat-straw lignin and also by the demonstration of sugar incorporation during the synthesis of lignin by oxidation of lignin precursors in the presence of sugars (40, p. 82).

Kertesz (46, p. 77) referred to the possibility that the protopectin insolubility is due to the formation of double salts of the polygalacturonic acids. Polyvalent cations may link carboxyl groups of different polygalacturonic acid chains. Crosslinking through polyvalent cations between the carboxyl groups of different pectin micelles, or between pectins and proteins is a possibility, as suggested by Owens et al. (74).

The addition of calcium salts improves the firmness of canned vegetable products in which a considerable quantity of the pectic

substances present have a low degree of esterification. This is true for tomatoes that are firmed by treatment with calcium chloride (52). Hoogzand and Doesburg (36) found that the firmness of canned cauliflower could be improved by a low-temperature long-time blanch in calcium chloride solution. They reported that the pectinesterase of the cauliflower tissue deesterified the pectins present sufficiently to cause them to be firmed by the calcium ion. In peas, phytic acid may be involved in calcium uptake rather than pectic acid (40, p. 84).

Pectic Enzymes

PECTINESTERASES (PE)

Action and specificity

Pectinesterases hydrolyze the methyl ester groups of pectic substances (19, p. 358). The action of these enzymes results in the production of pectinic and/or pectic acids from pectin with the liberation of equivalent amounts of methanol. Pectinesterases are highly specific enzymes which hydrolyze almost exclusively the methyl ester groups of pectic substances (19, p. 358).

Several types of methods have been used for the determination of pectinesterase activity. Determination of PE activity by the increase in free carboxyl groups was first described by Kertesz

(42). Another assay involves determination by estimation of the methanol. The methanol split off by the enzyme is distilled and determined, usually by means of colorimetric methods (46, p. 363). Other methods (46, p. 361) use the increase in sensitivity of the pectin toward electrolytes (coagulation, gelation).

Preparation and purification

The bulk of pectinesterase is usually strongly adsorbed on water-insoluble cellular constituents (46, p. 366). Pectinesterase can be desorbed from water-insoluble cellular tissues by the use of salt solutions, by raising the pH of the tissue macerate above 5.0 or by a combination of these two conditions. This enzyme is rather sensitive to exposure to pH values under 3.0 and above 8.0 (46, p. 367). Tomato fruit pectinesterase behaves differently from the orange, tobacco and alfalfa enzymes inasmuch as it is precipitated from solution during dialysis (61).

Effect of pH on activity of pectinesterase

The activity of pectinesterase of plant origin is affected by the pH of the reaction mixture and the salt concentration as well as by the cation component of the salt (51). The effect of the salts is to lower the pH at which maximum activity is attained and to extend

the activity into lower pH regions. In the range of pH 7.0 - 8.0 salts have little or no effect on the activity. In salt-free solutions the activity of pectinesterase of plant origin was nearly zero at pH 4.0 and increased as the pH was raised to 8.0 (46, p. 369; 51). Fungal pectinesterase is active in a salt free solution at pH 4.0 and has an optimum activity at about pH 4.5 (46, p. 370). The optimum pH for the pectinesterase of Penicillium chrysogenum was found to be 3.3 to 3.4 (76).

Salt activation

With divalent cations and at pH 6.0, maximum activation is produced in the neighborhood of 0.03 M concentration. At higher salt concentrations and the same pH, the activity is suppressed (46, p. 370). Monovalent cations usually produce maximum activation at pH 6.0 in 0.10 M concentration and do not suppress activity below molalities of 1.0.

The salts prevent the inhibition of the enzyme by the free carboxyl groups present in the pectinic acid substrate and also formed during the reaction (51). The cations are assumed to form cation-carboxyl complexes, thus eliminating the inhibitory effect.

The pectinesterase of higher plants is unusually resistant to the effect of chemical agents (59). Formaldehyde, iodine, iodoacetic

acid, hydrogen cyanide, and mercuric chloride are noninhibitory in the presence of the substrate. Soap solutions and detergents inactivate the enzyme (59).

The mold enzyme is active in a salt-free medium and the effect of added salt is primarily to increase the activity at the pH optimum (4.5 - 5.0) rather than to cause a change in the position of the optimum as is the case with the pectinesterase of higher plants (46, p. 372). At pH 7.0 no significant salt activation can be observed. Fungal pectinesterase is more resistant to chemical inactivation than the enzyme of higher plants. Detergent concentrations which inactivate the plant enzyme do not affect the mold enzyme. The mold enzyne is not precipitated from a solution upon dialysis similar to tomato PE (59; 46, p. 372).

Temperature effect

The pectinesterases of higher plants do not show activity loss when heated to 55°C for one hour at pH 4.0 - 6.0 (59). Above 60°C the enzyme is gradually inactivated. The tomato enzyme is heated above 80°C before inactivation (44). Under the same conditions the enzyme found in oranges is somewhat more sensitive to heat than the tomato enzyme (46, p. 372). The pectinesterase of molds is more sensitive to heat than that of higher plants since the heat inactivation of the fungal enzyme is quite noticeable at 30°C (59).

Occurrence in higher plants

The pectinesterase of higher plants has been studied most extensively. MacDonnell et al. (55) found that orange pectinesterase was highly specific for esters of polygalacturonic acid. Tomato pectinesterase has been studied by several groups of investigators (59; 32; 78). The extraction from pulp is most efficient under slightly alkaline conditions and in the presence of salt of phosphate buffer (34). The pH optimum of tomato pectinesterase is about 7.5 to 7.8.

Bell et al. (12) compared the pectinesterase content of cucumbers and tomatoes during various stages of development. Whereas in cucumbers, the total pectinesterase content per fruit dropped slightly during development to full maturity, the pectinesterase content per fruit in tomatoes increased rapidly.

Pectinesterase activity has been demonstrated in pears, (18, p. 133), in apples (79), in tobacco leaves (35), in garlic and in onion extract (69).

POLYGALACTURONASES

Action and specificity

Polygalacturonases hydrolyze the α-1, 4-glycosidic linkages of pectic substances (19, p. 361). Polygalacturonases are regarded as hydrolases in which a double displacement mechanism may be operative. According to this mechanism, the substrate is first attacked by a nucleophilic group of the enzyme and subsequently the enzyme-substrate intermediate is attacked by the nucleophilic water molecule (71, p. 190; 19, p. 361). There are different types of substrate breakdown and these types have been attributed to different polygalacturonases (18, p. 119). Two major criteria are taken into account in the classification of polygalacturonases: the substrate preference of the enzyme and the mechanism of attack. The optimum pH values of the enzymes have been used to a limited degree in the classification (18, p. 120). It has been found that most of the pectic enzymes "prefer" either pectin or pectic acid as substrate. This preference of the enzyme for the esterified or the unesterified form of pectic substance has been chosen as one of the major criteria for the enzyme classification. Random degradation of the chain is the most common type of breakdown encountered (18, p. 120). However, a terminal mechanism has also been found during

which galacturonic acid is liberated at the start of the reaction. This characteristic provides a second classification criterion. The prefix "endo" has been applied to those enzymes carrying out random hydrolysis and the prefix "exo" to the enzymes causing terminal hydrolysis (18, p. 120). The following polygalacturonase groups are recognized (18, p. 121).

Endopolymethylgalacturonase: This enzyme catalyzes a partial hydrolysis of pectin. About 25 percent of the total glycosidic bonds are broken yielding intermediate polyuronides, of 4 to 5 residues per molecule, as a product (88). The enzyme depolymerase belongs to this group (18, p. 121).

Endopolygalacturonase: It carries out a random hydrolysis of pectic acid to the 70 percent level resulting in a mixture of digalacturonic and galacturonic acids (54). Pectin cannot be attacked by the enzyme. It is of interest that preparations of pure endopolygalacturonase can also macerate certain types of native plant pectin (73). This would indicate that in maceration of plant tissues, a specific protopectinase is not required (18, p. 123).

Exopolymethylgalacturonase: Matus (18, p. 124) working with a commercial fungal preparation, studied the action of exopolymethylgalacturonase on pectic acid and on the glycol esters of pectic acids possessing various degrees of esterification. Glycol esters

were used, rather than naturally occurring methyl esters, so that accompanying pectinesterase could not influence the results, since pectinesterase cannot deesterify glycol esters. Matus (18, p. 125) found that as the degree of esterification was increased, the ratio of enzymic activity, in terms of liberated reducing groups to enzymic activity in terms of viscosity, increased. The highly esterified compounds appeared to be attacked from the end of the chain while random hydrolysis was the mechanism of pectic acid attack. Pectin was attacked at an optimum pH of 3.9.

Exopolygalacturonase: The hydrolysis of pectic acid goes to completion via galacturonic acid liberation from the end of the chain. The activity optimum lies within the pH range of 3.0 to 4.0. Brooks and Reid (14) have obtained exopolygalacturonase from the complex of pectic enzymes produced by Aspergillus foetidus. Exopolygalacturonase was found by Brooks and Reid (14) to be quite stable. They could adsorb it on Fuller's earth at pH 5.0. The optimum activity was found to be at pH 3.0 to 3.5.

"Mold polygalacturonase", whose action was considered for years to be the sole form of glycosidic hydrolysis of pectic substances, is absent from the above classification. Mold polygalacturonase is considered to be a mixture of endopolygalacturonase and exopolygalacturonase (18, p. 126).

Determination of polygalacturonase activity

The activity may be followed by determining the loss of substrate, by estimating the reducing groups formed or by measuring the physical changes which occur (46, p. 339). In measurements based on the loss of substrate the pectic substances are precipitated with ethanol or acetone, or as pectic acid or calcium pectate. The method has drawbacks such as the time-consuming nature of the precipitation and filtration (46, p. 340).

Polygalacturonase catalyzes the hydrolysis of 1, 4-glycosidic linkages in pectic substances. Every fissure of such a bondage produces a reducing group which was previously engaged in the formation of the polymer. The hypoiodite method of Willstätter and Schudel (46, p. 340) is used for this determination.

Viscosity changes have been extensively used in the estimation of polygalacturonase activity. The major viscosity changes occur very early in the reaction. After about one-fourth of all glycosidic linkages have been hydrolyzed, the viscosity remains relatively constant with further degradation. For this reason the measurement of viscosity changes provides a sensitive measure of even traces of polygalacturonase activity (46, p. 341; 83).

Changes in the optical rotation have been used to follow polygalacturonase action (46, p. 342). The optical rotation of a solution diminishes as pectinic acid is hydrolyzed. The change in rotation is slow in the early phase of hydrolysis (46, p. 342).

Preparation and purification

Procedures used for the isolation of polygalacturonase from the commercial enzyme preparations are discussed by Kertesz (46, p. 351). Chromatographic techniques used for pectic enzyme separation will be discussed later in this review.

Effect of pH and temperature

The pH of optimum activity of fungal polygalacturonase has been found to be in the neighborhood of 3.5 (46, p. 353). The literature (18, p. 122) contains data which indicate optimum ranges from 3.0 to 5.0. In general the pH optimum of PGs from ascomycetes lies near 4, that of PGs from bacteria and phycomycetes near 7 (46, p. 353).

Fungal polygalacturonase in the form of a dry powder is quite resistant to heat while polygalacturonase in water solution is less stable. When solutions are kept at 55°C for 30 minutes at pH 4.0 or for three hours at 50°C complete loss of activity has resulted

(46, p. 354). The resistance to heat is much enhanced by the addition of small amounts of sodium alginate, gelatin, glycerol and of the glycerol and glycol esters of pectic acid (18, p. 121). The temperature of optimum activity depends on the length of reaction time (71, p. 123). The temperature range for maximum polygalacturonase activity is from 30° to 50°C (46, p. 355).

Activation, inhibition and inactivation

It is not certain that the addition of electrolytes either increase or decrease the enzymic activity (46, p. 355; 19, p. 365). The effect of polyvalent ions on polygalacturonase activity is further complicated by the effect of such ions on the solubility of pectic and pectinic acids.

Bell et al. (10; 11) have demonstrated the presence of naturally occurring polygalacturonase and cellulase inhibitor in grape leaves. This inhibitor is a tannin-like substance which is precipitated by caffeine, nicotine sulfate and gelatin. The molecular weight of the tannin inhibiting polygalacturonase was found to be higher than 10,000 (80). Polygalacturonases produced by plant pathogens can be inactivated in rotted tissue by compounds formed during the oxidation and polymerization of plant polyphenols (101).

Occurrence in higher plants

Polygalacturonases are found in many bacteria and fungi (86). These enzymes have been found in higher plants as well, although in very low concentrations, i.e. in citrus fruit (19, p. 365). In tomatoes (68) a mixture of exo- and endopolygalacturonase exists in amounts varying according to the source of the tomatoes. Tomato polygalacturonase exhibits optimal activity at pH 4.5 (60). Claims of unusual heat resistance (60) were not confirmed in other reports (77).

McCready et al. (68) included avocado polygalacturonase in their studies. Avocado PG was found to be similar to the tomato PG preparation.

Weurman (18, p. 130) demonstrated the presence of a pectolytic enzyme in pears. Bell (8) studied the pectolytic activity of cucumbers, based on the loss in viscosity of a pectate solution. The seeds, flowers and ripe fruit were strongly active, but the enzyme was not found in the leaves, stems and in various stages of the immature fruit. It is not absolutely certain that the polygalacturonase is a natural constituent of the higher plants. Kertesz (46, p. 347) stated that he failed to produce conclusive evidence that polygalacturonase is a natural constituent of apple fruit.

MISCELLANEOUS PECTIC ENZYMES

Protopectinase: This enzyme was originally thought to hydrolyze the parent pectic substance, protopectin, producing soluble pectin (46, p. 334-337). The rapid softening of the fruit of Mespilus germanica attributed to protopectinase activity has not been confirmed (85). It is possible that protopectinase does not exist and the maceration of plant tissue can actually be carried out by certain known pectic enzymes (18, p. 128). Apparently some polygalacturonases and pectinesterases can attack protopectin (46, p. 303).

Pectin transeliminase: In a commercial polygalacturonase preparation an enzyme has been found which attacks only the methyl ester of pectic acid (2). A study of the breakdown products by ultraviolet absorption, ozonization and reaction with thiobarbituric acid indicated the presence of 4.5 unsaturated galacturonic acid groups (2). The enzyme seemed to proceed by a transelimination mechanism, similar to that previously observed for neutral and alkaline degradation of pectin (Figure 3). The enzyme was designated as pectin transeliminase.

Nagel et al. (70) studied the degradation of oligogalacturonides by the polygalacturonase of Bacillus polymyxa. Hydrolysis of trigalacturonate yields the altered dimer and monogalacturonate,

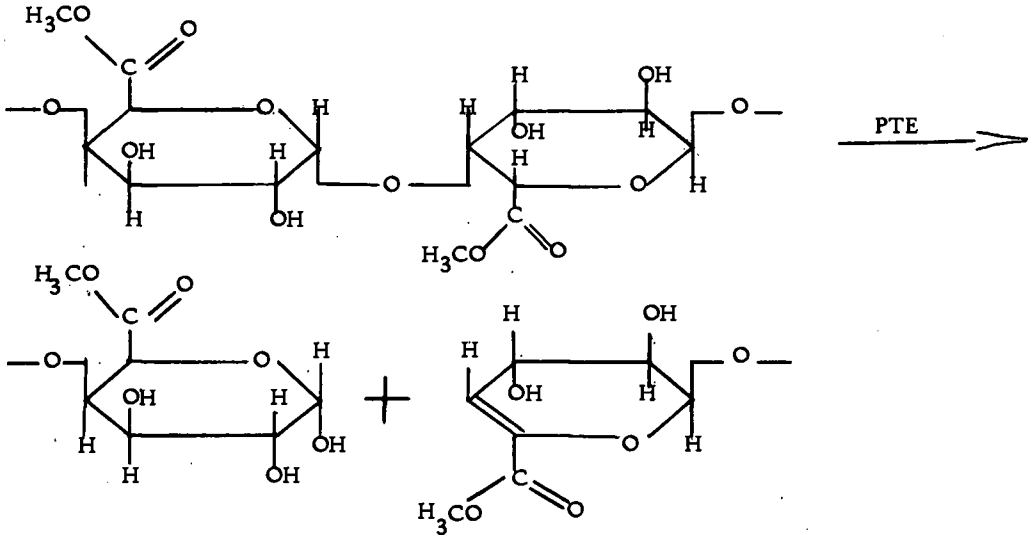


Figure 3. Mechanism of pectin transeliminase action.

while hydrolysis of the tetramer yields monomer and an altered trimer and, at a slower rate, normal dimer, and an altered dimer. The altered dimer has an α, β unsaturated bond.

Albersheim and Killias (3) described a procedure for the isolation and purification of fungal transeliminase and of transeliminase from the acetone powder of pea seedlings.

Separation of Pectic Enzymes

Limited heating of a preparation containing pectinesterase and polygalacturonase leads to complete destruction of pectinesterase but only to a partial inactivation of polygalacturonase (46, p. 374).

McColloch and Kertesz (58) have used a cation exchange resin (Amberlite IR-100) for the removal of pectinesterase from

commercial enzyme preparations.

Albersheim et al. (3) described a procedure for the purification of fungal pectin transeliminase using column chromatography on DEAE-cellulose. The solvent was 0.1 M phosphate-citrate buffer at pH 5.2. Elution was achieved by a continual increase in the NaCl concentration from 0 to 0.5 M. The transeliminase was further purified by the use of Sephadex-75 with 0.05 M phosphate-citrate buffer at pH 4.5 as the eluant.

McClendon and Kreisher (57) used cellulose phosphate for the separation of enzymes of the plant cell wall, which included the pectic enzymes. A pH gradient elution covering the range 2.2 to 7 was employed. The major peak of polygalacturonase was eluted at pH 6.0. Another peak appeared at pH 3.9 - 4.0. The starting material was extracted from the fungus, Rhizopus tritici.

McClendon and Hess (56) showed the presence of a variety of polygalacturonases in commercial preparations by gradient pH elution from columns of cellulose phosphate.

Effect of Enzyme Activity on the Texture of Vegetable Products

During the ripening of fruits the insoluble pectic substances are partially converted into a soluble form. The action of a specific enzyme, protopectinase, has been suspected, but no one has

succeeded in isolating such an enzyme (24). Other investigators (40, p. 40) have suggested the possibility that the hypothetical protopectinase and polygalacturonase are identical and that the solubilization of pectic substances is brought about by polygalacturonase. Kertesz (45) has indicated the possibility of a disintegration of protopectin by the action of ascorbic acid and hydrogen peroxide. A mechanism for the transformation of pectic substances of fruits has not been clearly demonstrated according to Joslyn (40, p. 2). Kertesz et al. (47) found that the firmness of apples correlated more closely with alcohol-insoluble solids content or cellulose content than with pectin content. Evidence is available that the amounts of cellulose and hemicellulose decrease during the ripening of apples (47) and peaches (94). The thinning of cell walls during maturation may be due to the degradation of cellulose as well as the pectic components, according to Joslyn (40, p. 33).

As a result of the action of polygalacturonase, a shortening of the chain lengths of the pectin molecules would be expected. When a partial breakdown (shortening) of chain length does occur, it is not always demonstrated by ordinary quantitative methods employed for the analysis of pectic substances (24). To detect such a partial breakdown it is necessary to use a method which gives an estimation of chain length, i. e. the viscosimetric method.

In 1954 McCready and McComb (67) reported the presence of polygalacturonase activity in peaches, pears and avocados. No polygalacturonase activity could be demonstrated in the unripe fruits, but ripe pears and avocados showed activity of 0.001 and 0.035 millimole of bonds split per hour per gram of fruit. Apparently the pectic enzymes come in contact with the pectic substances during ripening and hydrolyze them to materials of smaller molecular size. The degraded pectic materials are less effective in maintaining structure in fruits and less important in contributing to the consistency of processed fruits.

An investigation has been carried out on the changes in the amount and composition of the pectic substances during the storage of apples (37, p. 341). The changes follow a similar pattern irrespective of variety. The temperature affects the rate of change of the pectic substances, but does not affect the general trend which is a decrease in net protopectin accompanied by a rise in net water-soluble pectin. Later there was a period in which both fractions remained constant. As the fruit became mealy, soluble pectin decreased rapidly and protopectin again increased. The protopectin increase is not always obvious. It appears that softening in storage is due to the conversion of the insoluble pectic substances into soluble forms (37, p. 341).

Dame et al. (15) demonstrated conversion of protopectin into water-soluble pectin and hydrolysis of the water-soluble pectin to low molecular weight uronides in canned pears. Date and Hansen (16) also demonstrated conversion of protopectin to soluble pectin during softening of pears.

Woodmansee et al. (102) reported that a characterization of the total and soluble pectins showed that ripening of the apples and tomatoes was associated with a decrease in the apparent equivalent weight and in the degree of esterification of the pectins.

Postlmayr et al. (81) worked on the characterization of pectin changes in freestone and cling stone peaches during storage. Pectic constituents of Halford clingstone peaches changed slightly during maturation. High retention of protopectin is apparently the major factor responsible for the firm texture of clingstone peaches. In the case of Fay Elberta freestones, protopectin was converted into water-soluble pectin during ripening, causing a softening of the texture. A partial conversion of protopectin into water-soluble pectin occurred during heat processing of both varieties. Kertesz (43) indicated that pectic materials contribute to both the consistency and thickness of tomato juice and tomato catsup. A decrease in pectic constituents contributed to liquifaction and subsequent deterioration. During storage, pectic substances in these products were

hydrolyzed, unless the pectic enzymes were inactivated by proper heat treatment.

According to Stier (95) protopectin showed a significant decrease during the storage of fresh tomatoes.

McColloch et al. (62) reported that in tomato juice manufacture, the freshly crushed tomato fruit must be heated to 180° F (82°C) in order to inactivate the pectic enzymes. If temperatures lower than this are employed the enzymes remain active and alter the pectic substances and the resulting juice is thin and watery.

Gallop (27) found out that loss of water-soluble pectin was marked in strawberries, while conversion of water-soluble pectin into versene-soluble pectin was the major change which occurred in the pectic components of tomatoes when processed.

Van Buren et al. (100) studied the effect of pectin methylesterase in snap beans. The esterase which is believed to be relatively inactive in most plant tissues, rapidly converts pectins to pectic acid when the tissues are ground or macerated. The action of the enzyme results in a decreased solubility of the pectic substances, particularly in the presence of calcium salts and an increased firmness of tissues. The firmer beans had more pectic acid or pectate than the softer beans. Moderate blanching temperature favored the action of the enzyme.

Sistrunk and Cain (91) reported in 1960 that most of the texture changes in canned green beans appeared to be related to change in pectic substances during blanching and canning.

Staden and Doesburg (93) studied the disintegration of strawberry pulp preserved by means of sulphurous acid and calcium bisulphite. Pectolytic enzymes are not checked by the sulphurous acid or sulphites and are free to act on the pulp. It may be assumed that the enzymes are formed by either the strawberries or microorganisms. There is evidence (93) that these enzymes are not of microorganism origin. It is not easy to establish the origin of the pectolytic enzymes. The higher the pH of the pulp, the more pronounced was the disintegration. At pH 2.0 to 1.5 there is disintegration of texture caused by a chemical decomposition of the cell wall components. A pH value of 2.5 to 3.0 appeared to be optimum for the retention of a good consistency.

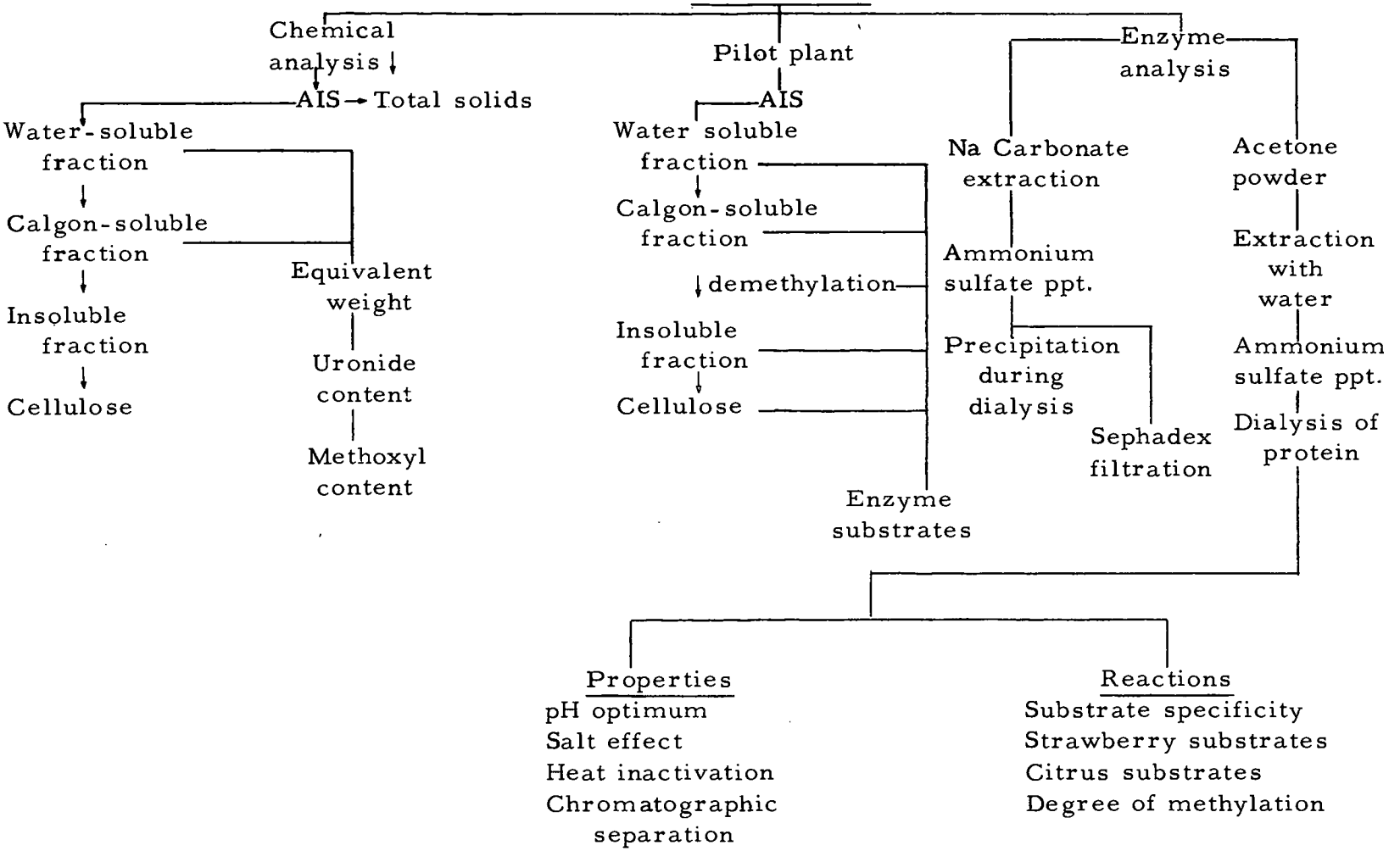
Cellulase

Reese (82) reported in 1956 that filtrates from cellulolytic microorganisms have little action on cotton that has not been made more accessible by swelling or partial degradation.

Selby et al. (89) reported in 1963 that there exist several cellulases some of which preferentially attack substrates of short

chain length whereas others are more active towards materials of higher degrees of polymerization. Agarwal et al. (1) found that ethyl malomate at low concentrations (0.05 percent) enhances cellulase activity of the enzymic system of the fungus, Chaetomium globosum, whereas at higher concentrations (0.50 - 0.75 percent) almost complete inhibition occurred. King et al. (49) found that several components of the commercial Aspergillus niger cellulase are distinctly different enzymes in terms of the mode of attack on cellulose substrates. Storvick et al. (96) investigated the mode of action of one of four electrophoretically distinguishable β(1-4) glucan hydrolases from Cellvibrio gilvus. Analysis of the intermediates indicated preferential attack at the second and third glucosyl bonds from the nonreducing end of the polysaccharide chain.

Graphic Flow Sheet of This Study



The experimental work of this study consisted of isolating the pectinolytic and cellulolytic enzymes in strawberries and the determination of the natural substrates upon which these enzymes act. The objective was the development of a theory to explain the textural changes in fresh and processed strawberries.

The enzymes were isolated by precipitating the strawberry solids with acetone, extracting the acetone powder with distilled water, precipitating the proteins with ammonium sulfate and dialyzing the water solution of this precipitate. This dialyzed protein solution was the enzyme preparation used for testing the properties of the pectinolytic and cellulolytic enzymes of strawberry.

Fractionation of the enzyme preparation was made using DEAE-cellulose chromatography.

The polygalacturonase activity of the isolated protein fractions was investigated by measuring the viscosity loss of a 1.0 percent solution of pectic substances in citrate buffer at pH 5.0 and a temperature of 30°C (86°F). The cellulolytic activity was investigated by measuring the loss in viscosity of a 1.0 percent carboxymethylcellulose solution in citrate buffer at pH 5.0 and a temperature of 30°C (86°F).

Source of the Fruit

Northwest strawberries used for this study were grown on Kiger Island south of Corvallis. Fruit used as a source of the enzyme was individually quick frozen at -27.7°C (-18°F) and kept under refrigeration at -20°C. This fruit was used for analytical work and for preparation of the acetone powder.

Analysis of the Strawberries

Total solids: After homogenizing in a Waring blendor for one minute, three 20 g samples of strawberry were weighed into 6 cm diameter aluminum foil dishes, dried on a steam bath until apparently dry, then dried under 29 inches vacuum at 60°C for 16 hours, allowed to cool in a desiccator for one hour and weighed (74).

Alcohol insoluble solids (AIS): The procedure described by Gallop (27, p. 54) was used for the determination of the AIS of the strawberries. Four 200 g samples of fruit were stirred into 800 ml of boiling 95 percent alcohol in a one liter beaker, the mixture quickly brought to the boil, and then held in a water bath at 80°C for 30 minutes. Each sample was then chopped in a Waring blendor, for 1/2 minute at half speed, followed by one minute at full speed,

washed back into a one liter beaker with 70 percent ethanol and heated in the 80°C water bath for 30 minutes.

The slurries were filtered through pre-dried, weighed, 12.5 cm, No. 31 Whatman filter papers, washed with 70 percent ethanol until the filtrates were colorless, drained on the Buchner, and each filter cake was separated from its paper before air drying upside down on the paper, then vacuum dried as for the total solids and weighed. The A.I.S. materials were ground in a Arthur H. Thomas Co. hammer mill, using the No. 80 screen and stored in screw-top glass jars. The ground samples were re-dried under 29 inches of vacuum at 60°C for 16 hours before subsequent analyses.

Extraction of water soluble pectins of the A.I.S.: A procedure similar to that described by Dietz and Rouse (21) was used. Samples of ground A.I.S., 0.2 g, were weighed into 50 ml conical centrifuge tubes. The material was rehydrated by the addition of 40 ml of water and the tubes heated in a boiling water bath for five minutes. The mixture was stirred and allowed to stand for one hour before being centrifuged. A second water extraction was made and the supernatant liquids decanted and combined.

Equivalent weight: The method used was similar to that of Owens et al. (74). To 0.5000 g samples of the pectic substances

moistened with five ml of ethanol, 100 ml of carbon dioxide-free water were added. The mixture was titrated slowly to avoid possible deesterification using 0.05 N sodium hydroxide to a pH 7.5 endpoint as determined by a Zeromatic pH meter.

$$\text{Eq. wt.} = \frac{1000 \times \text{wt. of sample (g)}}{\text{N} \times \text{vol of alkali (ml)}}$$

Methoxyl content: The method is described by Owens et al. (74). To the neutral solution titrated for equivalent weight (containing 0.5 g of pectic substance) 25 ml of 0.25 N NaOH were added. The solution was shaken thoroughly and allowed to stand 30 minutes at room temperature in a stoppered flask. Hydrochloric acid (0.25 N) equivalent to the base was added and the solution titrated with 0.05 N sodium hydroxide to pH 7.5.

$$\% \text{ MeO} = \frac{\text{N} \times \text{vol of alkali (ml)} \times 3.1}{\text{wt of sample (g)}}$$

Determination of water-soluble pectin: The pectin solution from the methoxyl determination was made to volume in a 250 ml volumetric flask. One ml aliquots of the deesterified pectin solutions were pipetted into 6" x 1" Pyrex test tubes, and 0.3 ml of 0.1 percent alcoholic carbazole was added to each tube. One-half ml of purified ethanol was used in the blank determination, instead of the carbazole solution. Six ml of concentrated sulfuric acid were added to each of the tubes with constant agitation. The tubes were rubber

stoppered and the color allowed to develop for ten minutes before transferring to 1/2 inch cells. Fifteen minutes after adding the acid, absorbance measurements at 525 mμ were made on a Bausch and Lomb, Spectronic 20 spectrophotometer. A standard curve was run with vacuum-dried, Eastman, Practical grade, d-Galacturonic acid, by the same procedure.

Sodium hexametaphosphate-soluble pectins: Twenty-five ml of water and ten ml of 4 percent sodium hexametaphosphate solution were added to the residue in each tube remaining after the extraction of the water-soluble pectins, the mixture stirred and allowed to stand for 60 minutes. The tubes were centrifuged at 2,500 rpm for 10 minutes, decanted and the residues extracted once with 40 ml of water. The two extracts were combined and made up to 100 ml before pectin analysis by the carbazole procedure.

Determination of the insoluble pectin: Thirty ml of boiling two percent sodium hydroxide were added to the residues and heated with stirring in a boiling water bath for 30 minutes, cooled and centrifuged. The liquors were decanted into 250 ml flasks. The residue was washed twice with 30 ml portions of water and the wash water decanted into the 250 ml flasks after centrifuging. The extracts were made up to volume and the extracted material was determined by the carbazole method.

Determination of cellulose: Thirty ml of acetone were added to the residue in each tube, heated in a water bath at 55°C for 30 minutes with stirring and then centrifuged. This lipid-extracting procedure was repeated once more and the residues were allowed to dry overnight at 25°C. One ml of water was added to each tube, and the tubes were heated in a warm (60°C) water bath for 30 minutes with stirring to disperse the solids and allowed to cool. Twenty-five ml of 60 percent sulfuric acid were added and the mixture was allowed to stand for three hours, with occasional stirring. The solutions were poured into 100 ml of water in 250 ml volumetric flasks and the tubes were washed out into the flasks with water. The solutions were cooled, made up to volume, and filtered through No. 31 Whatman papers. One ml aliquots of sugar solution were pipetted into 6" x 1" Pyrex test tubes. One ml of five percent phenol solution was added to each tube and mixed. Blanks were prepared with one ml of water instead of the sugar solution. Five ml of 96 percent sulfuric acid were added to each tube from a burette so that the flow hit the liquid surface directly to produce mixing and even heat distribution. The contents of the tubes were mixed and after ten minutes the tubes were shaken and placed in a water bath at 30°C for 20 minutes. The absorbance was measured in a Spectronic 20 spectrophotometer at 490 mμ. Standard curves were run using glucose.

Precipitation, Purification and Fractionation of Strawberry Alcohol Insoluble Solids (AIS)

The work that follows was pilot plant study of the isolation of pectin substances from strawberry.

Precipitation and Purification of AIS: The strawberries were washed in a McLauchlan vibratory washer, sorted on an Allen vibrating table and drained on eight-mesh stainless steel screens. Twelve and seven tenths pounds of berries (random samples) were macerated in a Fitzpatrick Comminuting Mill, Model D, using the No. 2 screen. While the strawberries were macerated in the comminuting machine, sufficient 95 percent ethanol was added to the fruit passing through the machine to give a final alcohol concentration of 70 percent. This mixture was heated in a steam-jacketed kettle to 170°F and kept at this temperature for 30 minutes to achieve enzyme inactivation (74). After heating the insoluble solids were separated from the liquid by filtration through a nylon cloth. The solids were air dried, dispersed in water, and sufficient 95 percent ethanol was added slowly to bring the final ethanol concentration to 60 percent. After allowing the dispersion to stand overnight, it was again filtered through a nylon cloth. The solids, after being air dried, were again dispersed in water and acetone was added slowly to a concentration of 50 percent. After steeping for 12 hours the dispersion was filtered and redispersed in

acetone. Following the filtration from the acetone, the solids were air dried.

The AIS were fractionated by the procedures described in the following sections.

Extraction of the water soluble fraction of A. I. S. Five grams of the AIS and 200 ml of water were placed in a 200 ml centrifuge tube. The dispersion, after the addition of 2 ml toluene, was allowed to stand for 24 hours. The samples were centrifuged for 10 minutes at 2,500 rpm in an International Centrifuge, Model UV. The water extraction was repeated until the extract gave a negative anthrone test. Four extractions were needed. The water extract was originally freeze-dried but since this procedure resulted in a pectinate film, this step was discarded. Instead, the pectin was precipitated by the addition of 95 percent alcohol to a final alcohol concentration of 60 percent. The alcohol contained one percent hydrochloric acid. The mixture was allowed to stand for 12 hours, centrifuged for 10 minutes at 2,500 rpm, washed with 70 percent ethanol, with acetone, air dried and finally dried in a vacuum oven (29 inches) at 60°C for 24 hours. The dried material was ground in an Arthur H. Thomas Co. hammer mill, screen No. 80. This water-soluble pectin fraction was used as a substrate for checking the catalytic effect of strawberry proteins by the decrease in viscosity of the solution.

Extraction of the sodium hexametaphosphate-soluble fraction of A. I. S.: The residues from the water extraction were shaken with 200 ml of 0.4 percent sodium hexametaphosphate solution. The dispersion was allowed to stand for 24 hours and then was centrifuged for 10 minutes at 2,500 rpm. The extraction was repeated once more. The pectin was precipitated by the slow addition of ethanol, containing one percent hydrochloric acid, until a final concentration of 60 percent ethanol. The mixture was allowed to stand for 12 hours and centrifuged for 10 minutes at 2,500 rpm. The precipitate was washed with 70 percent ethanol, with acetone and dried in a vacuum oven for 24 hours at 60°C. The dried pectin was ground in an Arthur H. Thomas Co. (screen 80) hammer mill. This fraction was used as substrate for testing the pectinolytic activity of the strawberry proteins.

Insoluble residue of A. I. S.. The insoluble residue from the sodium hexametaphosphate extraction was dispersed twice in water to remove the sodium hexametaphosphate. Finally it was dispersed in acetone, centrifuged, air dried and dried in a vacuum oven for 24 hours at 60°C. The solids were ground in an Arthur H. Thomas Co. (screen 80) hammer mill. The residue was used for testing the activity of strawberry proteins.

Preparation and Viscosity Measurement of Strawberry Slurry

One hundred grams of strawberry were chopped in a Waring blendor for 1/2 minute of half speed, followed by one minute at full speed and the slurry was mixed with 0.1 M sodium chloride solution at a 1:1 ratio. One percent toluene was added to each sample as a bacteriostatic agent. The viscosity of the slurry was measured using a CaLab Capillary Viscometer. The viscosity is expressed in centipoises and calculated by using the formula:

$$\frac{\eta_1}{\eta_2} = \frac{d_1 t_1}{d_2 t_2} .$$

Where η_1, d_1, t_1 are the viscosity, the density and the flow time of the sample and η_2, d_2, t_2 the same quantities for distilled water at the same temperature.

Precipitation and Dialysis of Strawberry Proteins

Preparation of acetone powder: Fifty grams of strawberry were converted to pulp in a mortar. The pulp and five volumes of reagent grade acetone, precooled to -20°C, were blended at half speed for 1/2 minute and at full speed for one minute in a Waring blendor at -20°C. All equipment used was precooled to -20°C. The mixture was vacuum filtered through Whatman No. 31 filter

paper. The cake was blended with 100 ml of cold acetone, vacuum filtered and this washing step repeated. The acetone powder was dried under vacuum over sulfuric acid for 24 hours at room temperature and stored at -20°C (4). The yield was one gram of acetone powder from 63 g of fresh fruit.

The acetone powder was extracted with cold (1°C) distilled water in the ratio of 0.1 g acetone powder in 20 ml of water. The dispersion was allowed to stand for 30 minutes with occasional stirring. The solution made from the acetone powder was used for the precipitation of the proteins.

Precipitation of protein: After standing for 30 minutes the dispersion was filtered through Whatman No. 1 filter paper and ammonium sulfate was added to the filtrate, with stirring, to 75 percent saturation. The suspension was set aside overnight at 1°C, then centrifuged at 2,000 rpm for 10 minutes and decanted. The precipitated proteins were dissolved in a small amount of water and the solution was dialyzed for 14 hours against distilled water at 1°C in a cellophane dialysis tubing.

This dialyzed protein solution was used for the determination of the pectinolytic anc cellulolytic activity of the strawberry proteins.

Extraction of proteins with a sodium carbonate solution: The method is similar to the method described by Dewar et al. (20) for

the precipitation of the proteins from plant tissue. Two hundred and fifty grams of strawberry were chopped in a Waring blendor at half speed for 1/2 minute and a full speed for one minute and suspended in two liters of 0.025 percent cold (1°C) sodium carbonate solution. The dispersion was stirred from time to time and kept for 12 hours at 1°C. The mixture was centrifuged for 10 minutes at 2,500 rpm and decanted. Ammonium sulfate was added to the liquid, with stirring, to 75 percent saturation to precipitate the proteins. The amount of the salt added was calculated by the use of the nomogram of Dixon (23, p. 49). The mixture was set aside overnight at 1°C, after which the bulk of the supernatant liquid was decanted and discarded. The remainder was centrifuged, the precipitate dissolved in water and dialyzed in cellophane dialysis tubing. After dialysis of more than four hours, precipitation took place in the dialysis tubing and an alternate purification method was sought.

Instead of dialyzing, separation of the ammonium sulfate from the proteins by gel filtration was investigated. This method is based on the ability of Sephadex to exclude solutes of large molecular size and to be accessible for diffusion to molecules of smaller dimension. Sephadex-50 was obtained from Pharmacia Laboratories Inc., Rochester, Minn. A column 20 x 2 cm was used. The Sephadex-50 was packed for chromatography in accordance with the directions of the manufacturer.

Viscometric Determination of Polygalacturonase Activity

The method used was similar to that described by Bell et al. (9). A 1.2 percent solution of the pectic substances in sodium hydroxide-citric acid buffer at pH 5.0 was prepared. The buffer contained 2.0 g sodium hydroxide and 5.0 g citric acid per 1,500 ml of buffer. The viscosity was determined by the use of an Ostwald pipette in a Blue M water bath at 30°C (86°F). Ten ml of the substrate solution and two ml of the enzyme preparation were placed in the pipette and mixed. The final concentration of the pectic substances was 1.0 percent. The sodium pectate used was Sunkist sodium polypectate 6024 y 24.

Activity units proposed by Bell (9) are linear to enzyme concentration with 100 units of activity equaling 50 percent loss in viscosity of a 1.0 percent pectate-enzyme solution at 30°C, pH 5.0, for a 20-hour period. When the loss in viscosity value is plotted against the log of the enzyme concentration or of time the data are nearly linear (9). The percent loss in viscosity is calculated by the following formula.

$$\text{Percent loss in viscosity} = \frac{A - B}{A - W} \times 100$$

Where A is the initial flow time of the sample, B the flow time at the end of the period, W the flow time of water in the viscosity pipette. A conversion Table of percent loss in viscosity to pectinolytic activity is given by Bell (9).

Optimum pH of the pectinolytic activity: Citrate buffers were prepared as described in the previous section and the pH was adjusted to the desired value with 0.1 M sodium hydroxide or citric acid solutions using a Zeromatic pH meter. These buffer solutions were used for the determination of the optimum pH of the strawberry polygalacturonase.

Effect of salt concentration: To a citrate buffer, pH 5.0, prepared by dissolving 2.0 g sodium hydroxide and 5.0 citric acid in 1,500 ml distilled water, sodium chloride was added to give solutions with a concentration of 0.3 M and 0.6 M sodium chloride. The 1.2 percent sodium pectate solutions were prepared in these sodium chloride buffered solutions. Ten ml of substrate and 2 ml of enzyme solution were used for the viscosity determination so the final sodium chloride concentrations became 0.25 and 0.50 M. These salt solutions were used for the study of the effect of salt concentration on the activity of the polygalacturonase.

Heat inactivation of the pectinolytic activity: Five ml aliquots of dialyzed protein solution corresponding to 0.067 g of acetone

powder, prepared from the ammonium sulfate precipitate of the water extract of the acetone powder were placed in a 6" x 1" Pyrex test tube and stoppered. The tubes were immersed in a boiling water bath for either 20 or 35 minutes. The pectinolytic activity of the heated protein solutions was determined in an Ostwald pipette by calculating the percent loss in viscosity of a 1.0 percent sodium pectate solution in citrate buffer at pH 5.0 at 30°C (86°F).

Demethylation of calgon-soluble pectins: One and two-tenths grams of calgon-soluble pectin were blended in a Waring blendor with 20 ml of distilled water. Twenty-five ml of 0.25 N sodium hydroxide were added and the solution was allowed to stand for 30 minutes. Twenty-five ml of 0.25 N hydrochloric acid were added and the pH was brought to 5.0 by adding 0.1 M citric acid. The solution was made up to 100 ml by adding citrate buffer at pH 5.0. This fraction was used as a substrate for studying the activity of strawberry proteins.

Effect of the strawberry proteins on the hydrolysis of the insoluble residue of the strawberry A.I.S: Eight grams of the residue that remained after the extraction of the water-soluble and the sodium hexametaphosphate-soluble pectins were dispersed in 300 ml citrate buffer, pH 5.0. Toluene was added as the bacteriostatic agent at a level of one percent. To 150 ml of the above dispersion,

dialyzed protein solution (ammonium sulfate precipitated) equivalent to 0. 4 g of acetone powder was added, while the rest of the suspension was used as a blank, after the addition of distilled water equal to the volume of the protein solution added to the sample. Samples and blanks were kept at 36°C (97°F) under continuous stirring. Initially, and at time intervals, samples from the 150 ml dispersion were taken and filtered through Whatman No. 31 filter paper. One ml of the filtrate was diluted to 50 ml with distilled water in a volumetric flask and the solution was analyzed for carbohydrate content by the phenol-sulfuric acid colorimetric method.

Removal of the protopectin from the insoluble residue: Three hundred ml of boiling two percent sodium hydroxide were added to 8 g of insoluble residue and heated with stirring in a boiling water bath for 30 minutes, cooled and centrifuged at 2, 500 rpm for 10 minutes. The liquor was decanted. The same procedure was repeated until the liquor gave a negative anthrone reaction (two extractions). The precipitate was washed with 300 ml of distilled water, 200 ml acetone and was air dried. This precipitate was considered to be the cellulose fraction of the insoluble residue and the effect of strawberry proteins upon this fraction was investigated.

Commercial Enzymes

Pectinol-10 or Pectinase-D (0.05 g) were dispersed in cold (1°C) distilled water and the suspension was allowed to stand for 30 minutes. The insoluble material was removed by centrifugation at 2,500 rpm for 10 minutes. The liquid was dialyzed against distilled water at 1°C for 10 hours. The dialyzed liquids were used as the enzyme solutions for the investigation of the activity of commercial enzymes on the native strawberry pectins.

Assay Method for Pectinesterase Activity

The method described by Owens et al. (74) was used. Two ml of a 1.5 M sodium chloride solution were added to 10 ml of one percent pectin solution, stirred and titrated to pH 7.5 with 0.05 N sodium hydroxide. A constant temperature bath was used to maintain a temperature of 30°C. Enzyme preparation and water were added to adjust the volume to 20 ml and the pH was adjusted quickly to 7.5. The volume of alkali required to maintain the pH at 7.5 for 10 minutes was recorded. The concentration of the enzyme was adjusted to require about 0.5 to 1.5 ml of 0.05 N alkali in 10 minutes. The pectin used was Sunkist pectin N. F. No. 3442.

The results are expressed in meq of ester hydrolyzed per minute per 100 g of fresh tissue.

Extraction of pectinesterase with NaCl: Pectinesterase in most plant tissues is rarely in solution but is absorbed on the insoluble cellular solids (74). Twenty-five grams of strawberry were chopped at half speed for 1/2 minute and at full speed for one minute in a Waring blendor. The pulp was mixed with 25 ml of 0.25 M sodium chloride solution and the pH was adjusted to 8.0 and maintained at this pH for one hour. The mixture was filtered through Whatman No. 1 filter paper and 5 ml of the filtrate were used for the pectinesterase activity determination as described in the previous section.

Transeliminase activity: This method is described by Albersheim et al. (3). Changes in the light absorption at 235 mμ of a 0.5 percent citrus pectin solution in 0.1 M citrate-phosphate buffer, pH 5.2, after the addition of enzyme were followed. In the presence of enzyme the absorbance increases. The dialyzed solution of the ammonium sulfate precipitate of the water extract of acetone powder was used as the source of the enzyme. The buffer was prepared by mixing equimolar concentrations of dibasic potassium phosphate and citric acid and adjusting to pH 5.2. The pectin used was Sunkist pectin N.F. No. 3442. A unit of pectin transeliminase is defined as that

amount of enzyme in 0.1 ml of enzyme solution which will cause the absorption of light at 235 mμ by 2.0 ml of a 0.5 percent citrus pectin solution, contained in a 1 cm cuvette, to be increased by one optical density unit in 1 minute (3).

Viscometric Determination of Cellulase Activity

The method used was similar to that described by Bell et al. (9). A 1.2 percent solution of carboxymethyl cellulose - 7MP in sodium hydroxide-citric acid buffer at pH 5.0 was prepared. The buffer was prepared as described in the viscometric determination of the polygalacturonase activity. The technique described in the determination of the polygalacturonase activity was used for the determination of the cellulase activity.

One hundred units of cellulolytic activity represent a 50 percent loss in viscosity of 1.0 percent carboxymethyl cellulose-enzyme solution at 30°C, pH 5.0 for 20 hour period (9). Bell et al. (9) gave a table to make conversions from loss in viscosity to cellulolytic activity units. The cellulolytic units show a linear relationship to the enzyme concentration.

Chromatography of Strawberry Proteins on DEAE-Cellulose

The enzymes in the dialyzed protein solution of the ammonium sulfate precipitate of the water extract of the acetone powder were

separated by means of cellulose chromatography using DEAE-cellulose (cellulose N, N-diethylaminoethyl ether No. 7392), obtained from Eastman Organic Chemicals (3). Twenty grams of DEAE-cellulose were washed with 300 ml 1N sodium hydroxide, filtered in a coarse-sintered glass funnel and washed four times with 200 ml portions of water. The DEAE-cellulose was adjusted to pH 5.0 with 0.5 M citric acid and washed several times with citrate buffer, pH 5.0. The fines were discarded by suspending the cellulose twice in distilled water allowing the heavier material to settle and decanting the supernatant before the adjustment of the pH. The column used was 18 x 1.8 cm. The dialyzed protein solution from 1.5 g of acetone powder was placed directly on the DEAE-cellulose column. Fractions of 10 ml each were collected using a LKB 3400 B RadiRac fraction collector at 4°C. A sodium chloride concentration gradient was used for the elution. Fifty ml of citrate buffer, pH 5.0, were passed through the column initially followed by 50 ml portions of the same buffer made 0.1 M, 0.2 M, 0.3 M and 0.4 M with respect to sodium chloride.

RESULTS AND DISCUSSION

Introduction

The viscosity of a strawberry slurry was found experimentally to decrease with time. Enzymic activity or acid hydrolysis were thought to be possible causes of this decrease. To study this hypothesis the strawberry proteins were precipitated with acetone, purified by precipitation with ammonium sulfate, dialyzed and assayed for pectinolytic activity by measuring the decrease in the viscosity of 1.0 percent solutions of sodium pectate, citrus pectin, the water-soluble pectic fraction and the sodium hexametaphosphate-soluble pectic fraction of the Northwest strawberry. It was found that the dialyzed, strawberry, protein solution catalyzed a decrease in the viscosity of all the pectic solutions. While the viscosity decreased even in the absence of the protein preparation, a definite catalytic effect was attributed to the presence of polygalacturonase. The effect of pH and salt concentration on the activity of this enzyme and the rate of heat inactivation were investigated.

Besides the PG enzyme, the presence of pectinesterase and pectin transeliminase was investigated. Since there was pectinesterase activity, the pH optimum and the amount of this enzyme were studied.

Experiments with Strawberry Slurry

Decrease in viscosity: After maceration in a Waring blendor, strawberry pulp was mixed with 0.1 M sodium chloride solution at a 1:1 ratio and the viscosity measured at intervals using a CaLab Capillary viscometer.

Table 2 gives the viscosity of slurry samples stored at 22°C (72°F), 37°C (100°F) and 1°C(34°F) for different storage periods. Toluene, at the level of one percent, was added as bacteriostatic agent.

Table 2. Viscosity of strawberry slurries measured at 22°C (72°F). Means of six determinations

Storage time in hours	Viscosity in centipoises		
	Storage at 34°F	Storage at 72°F	Storage at 100°F
0	14.09	14.09	14.09
6	13.96	13.55	12.09
17	13.77	12.61	11.81
26.5	13.60	11.72	11.54
41	13.36	10.54	10.18
52	13.16	9.55	9.06
65	12.96	8.41	8.01
76.5	12.73	7.26	6.84
90.5	12.53	6.11	5.82

Figures 4, 5 and 6 show a linear change in the viscosity of the strawberry slurry as a function of time.

The change in the viscosity could be attributed to a hydrolysis of the hydrophilic molecules whose molecular weight is high enough to contribute to the viscosity of the slurry. Hydrolytic reactions

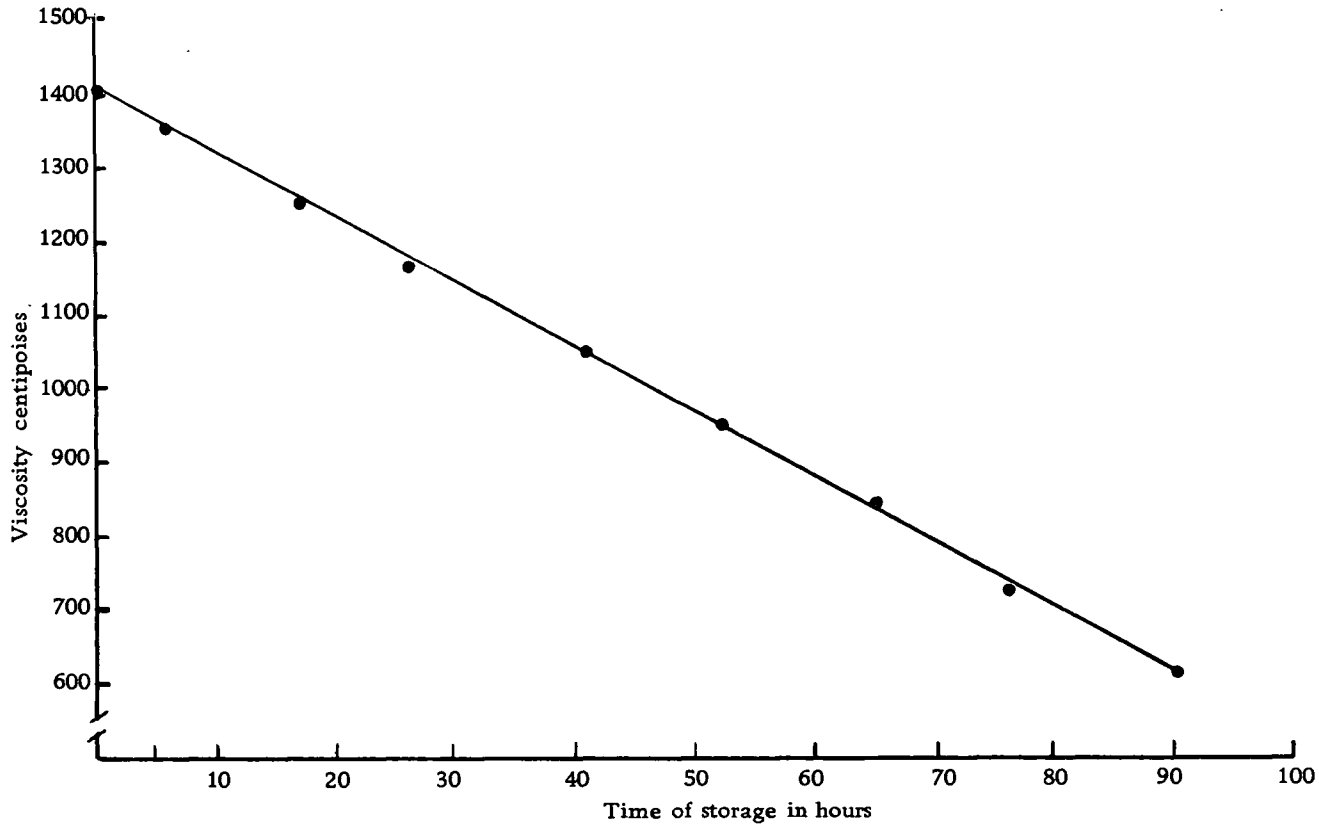


Figure 4. The viscosity of strawberry slurry stored at 72°F as a function of the storage time.

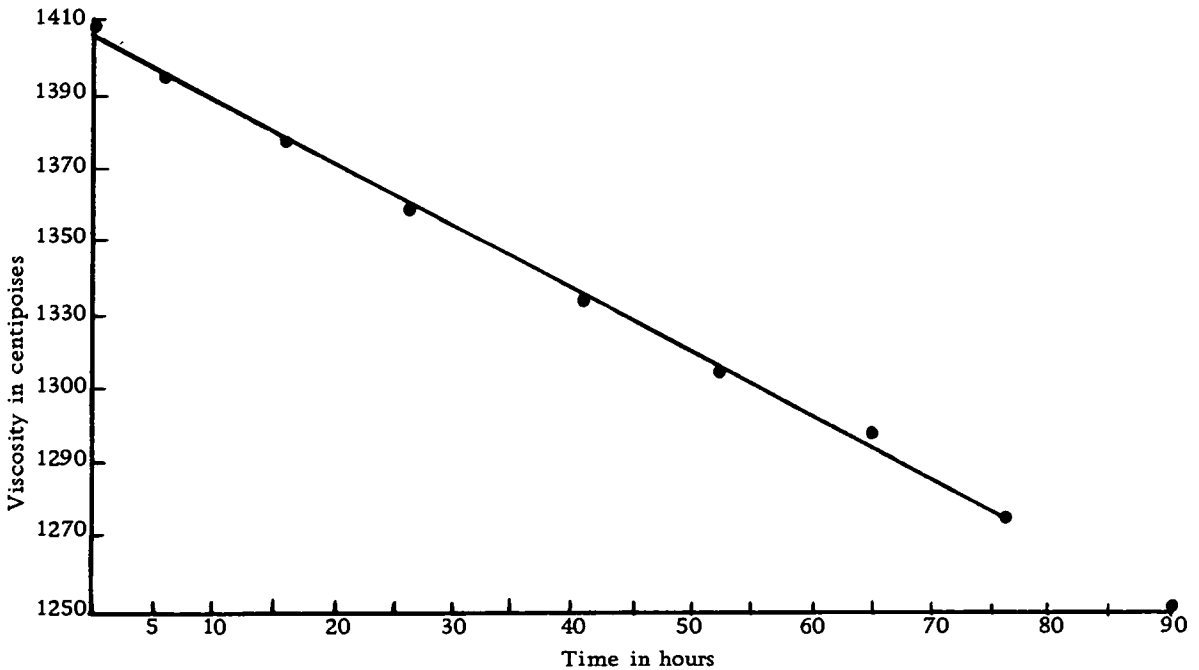


Figure 5. The viscosity of strawberry slurry stored at 34°F as a function of the storage time.

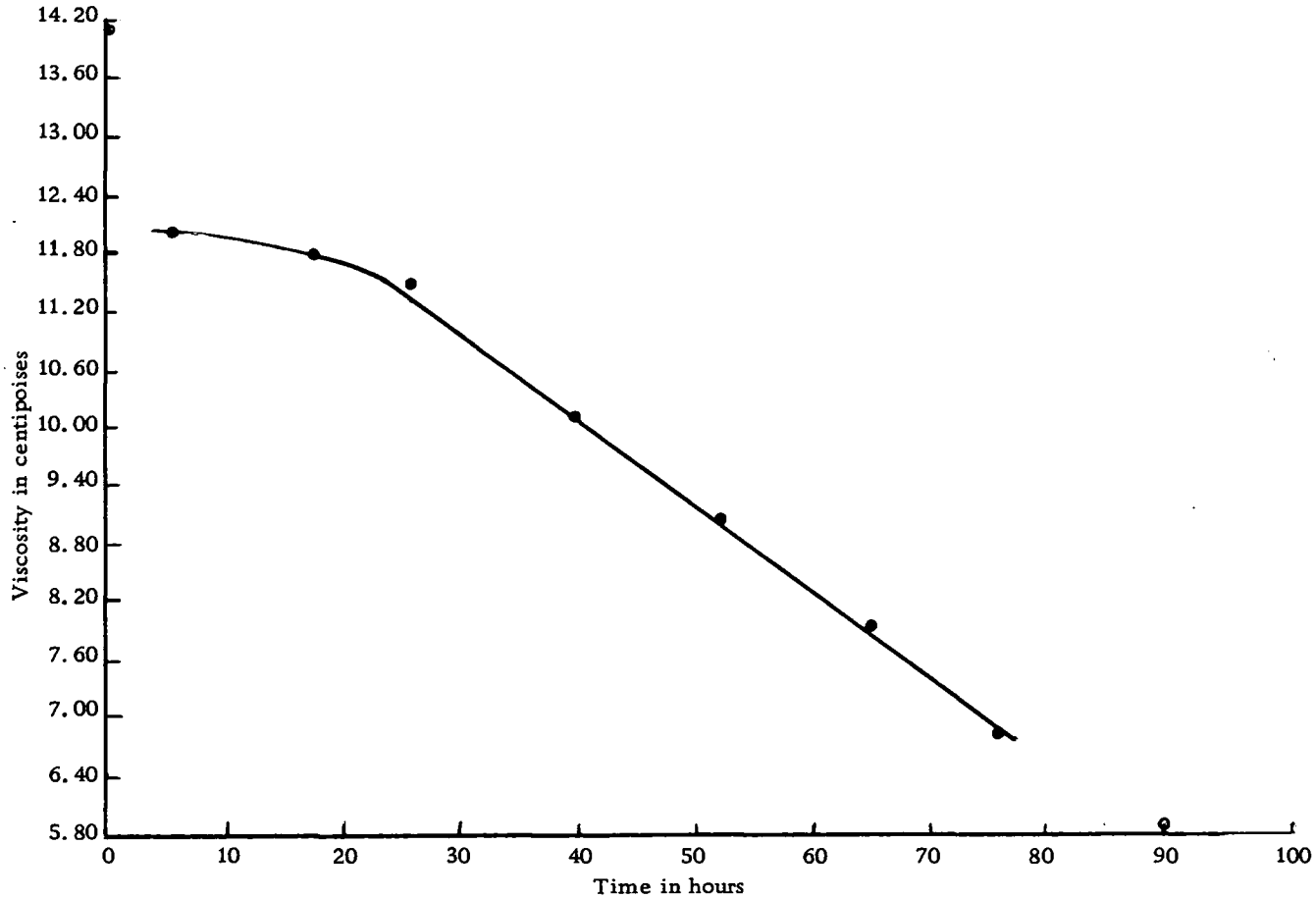


Figure 6. The viscosity of strawberry slurry stored at 100°F as a function of the storage time.

could be catalyzed by the organic acids present in the strawberry slurry, by native enzymes, by fungal enzymes or by any combination of these factors.

Since the pectic substances have a significant effect upon the viscosity of fruits, the possibility that the change in the viscosity of the strawberry slurry was related to changes in pectic substances was investigated using isolated and purified components.

Effect of heat on the slurry: The effect of heating on the decrease in viscosity of the strawberry slurry was studied. A strawberry slurry sample was heated at 212°F for 35 minutes and subsequently stored at 100°F together with a nonheat treated control sample. Toluene, at the level of one percent was added as a bacteriostatic agent and the viscosity of these samples was measured by the use of a CaLab viscometer at 32.2°C (90°F). The results are shown in Table 3.

Table 3. Viscosity of strawberry slurries heated at 212°F for 35 minutes.

Means of six samples

Storage time in hours	Viscosity in centipoises at 32.2°C (90°F) Storage at 37.7°C (100°F)	
	Heat treatment	No treatment
0	7.95	10.54
7	7.88	9.89
15.5	7.86	9.13
25	7.80	8.54

The strawberry slurry sample heated at 212°F for 35 minutes showed a significant initial change in viscosity, but the rate of the change during the storage at 37.7°C was lower than that of the untreated sample. The viscosity change during heating, which amounted to a 25 percent loss of the initial viscosity, could be attributed to accelerated chemical hydrolysis of macromolecules or to increased enzymic action during the initial stage of heating. The decrease in viscosity of the untreated sample during storage at 32.2°C (90°F) was 19 percent of the initial viscosity.

Precipitation and Dialysis of Strawberry Proteins

In order to check the possibility that enzymic activity contributes to the decrease in the viscosity of the strawberry slurry, the proteins of the fruit were precipitated and dialyzed for further study.

Two different methods for the precipitation of the proteins were used. One method consisted of precipitating strawberry solids with acetone at -20°C, extraction of the acetone powder with cold distilled water and precipitation of the proteins with ammonium sulfate. The other method consisted of extracting the tissue with sodium carbonate solution at 1°C and precipitation of the proteins with ammonium sulfate.

When the ammonium sulfate precipitate from the carbonate extraction was dialyzed against distilled water at 1°C for more than four hours, precipitation took place in the dialysis tubing. McColloch et al. (61) reported that tomato fruit pectinesterase was precipitated from solution during dialysis. A precipitation of strawberry enzymes upon prolonged dialysis is not unlikely. On the other hand, no precipitation took place during dialysis when the proteins were precipitated by ammonium sulfate from the acetone powder. Possibly a protective colloid exists in the acetone powder extract. Removal of the ammonium sulfate by gel filtration (Sephadex column) met with the difficulties of slow filtration rate (0.4 ml/hr) and the sample dilution by the eluting distilled water.

In the experiments that follow the dialyzed solution in distilled water of the ammonium sulfate precipitate of the acetone insoluble solids of strawberry tissue was checked for pectinolytic and cellulolytic activity.

Study of the Pectinolytic Activity of Strawberry Proteins

The decrease in the viscosity of a 1.0 percent solution of various pectic substances in citrate buffer, pH 5.0, at 30°C (86°F) was used to estimate the effect of the strawberry proteins on the hydrolysis of the pectic substances. Changes in viscosity would be

directly related to the consistency of the fruit while changes in the reducing power possess a more complex relationship with consistency. There is little change in reducing power during the early stage of the action of polygalacturonases.

Catalytic effect of the strawberry proteins on the pectic substances: Sodium pectate, citrus pectin, the water-soluble pectic fraction and the sodium hexametaphosphate-soluble pectic fraction of the strawberry alcohol insoluble solids were used as substrates for the investigation of the catalytic activity of the strawberry proteins isolated from the acetone powder. The effect of the proteins on the insoluble fraction of A. I. S. will be reported later in this work.

The results are given in Table 4.

Table 4. The percentage drop in viscosity of 1.0 percent substrate solutions, after 20 hours at 30°C.

Means of 10 determinations

Substrate	Protein added	Blank	Difference	Coefficient of variation	Pectinolytic units
Na pectate	26	6	20	1.9	1040 per g of acetone powder
Citrus pectin	23	3	20	1.9	
Water soluble fraction	12	7	5	4.0	
Calgon soluble fraction	16	7	9	2.0	

Figures 7 to 10 give the course of the viscosity changes of the substrates as a function of time. The amount of protein added to each sample corresponded to 0.025 g of acetone powder. In the blanks, water,

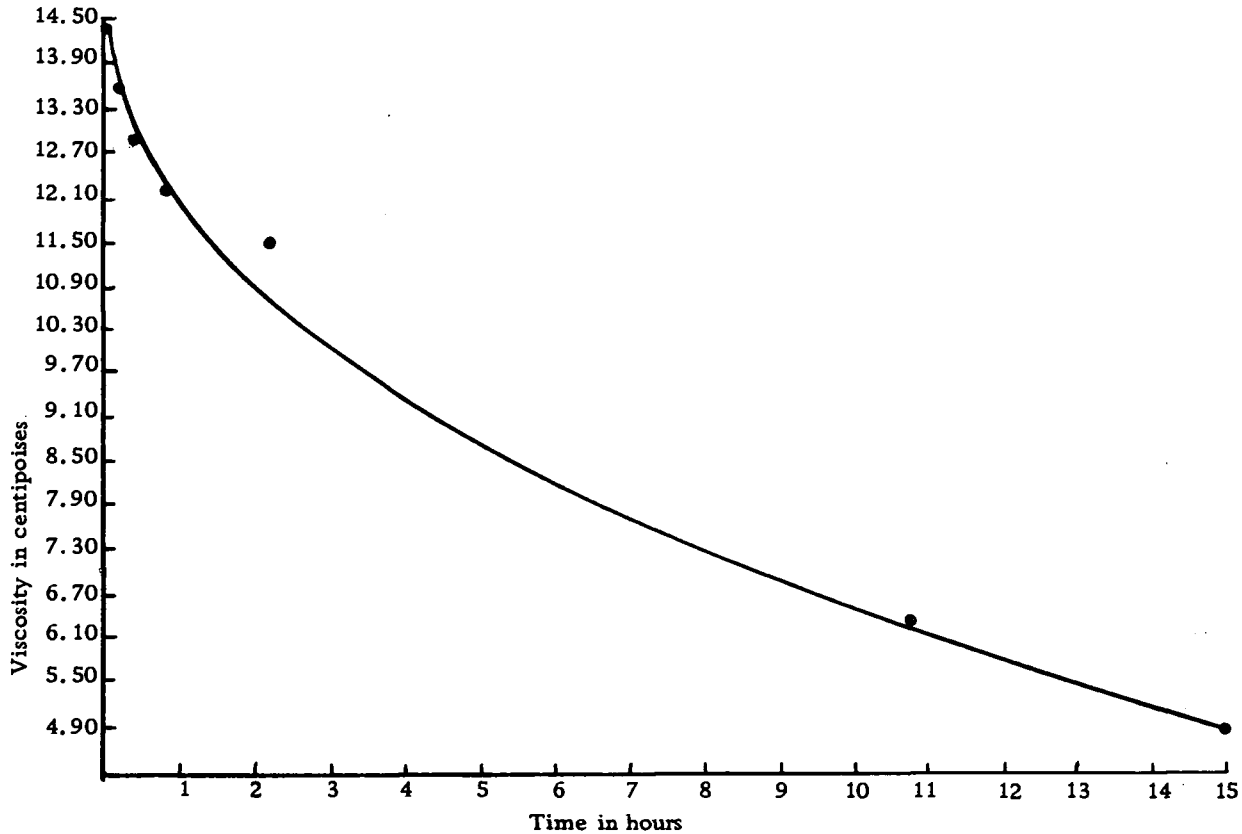


Figure 7. Course of action of strawberry proteins on pectate solution.

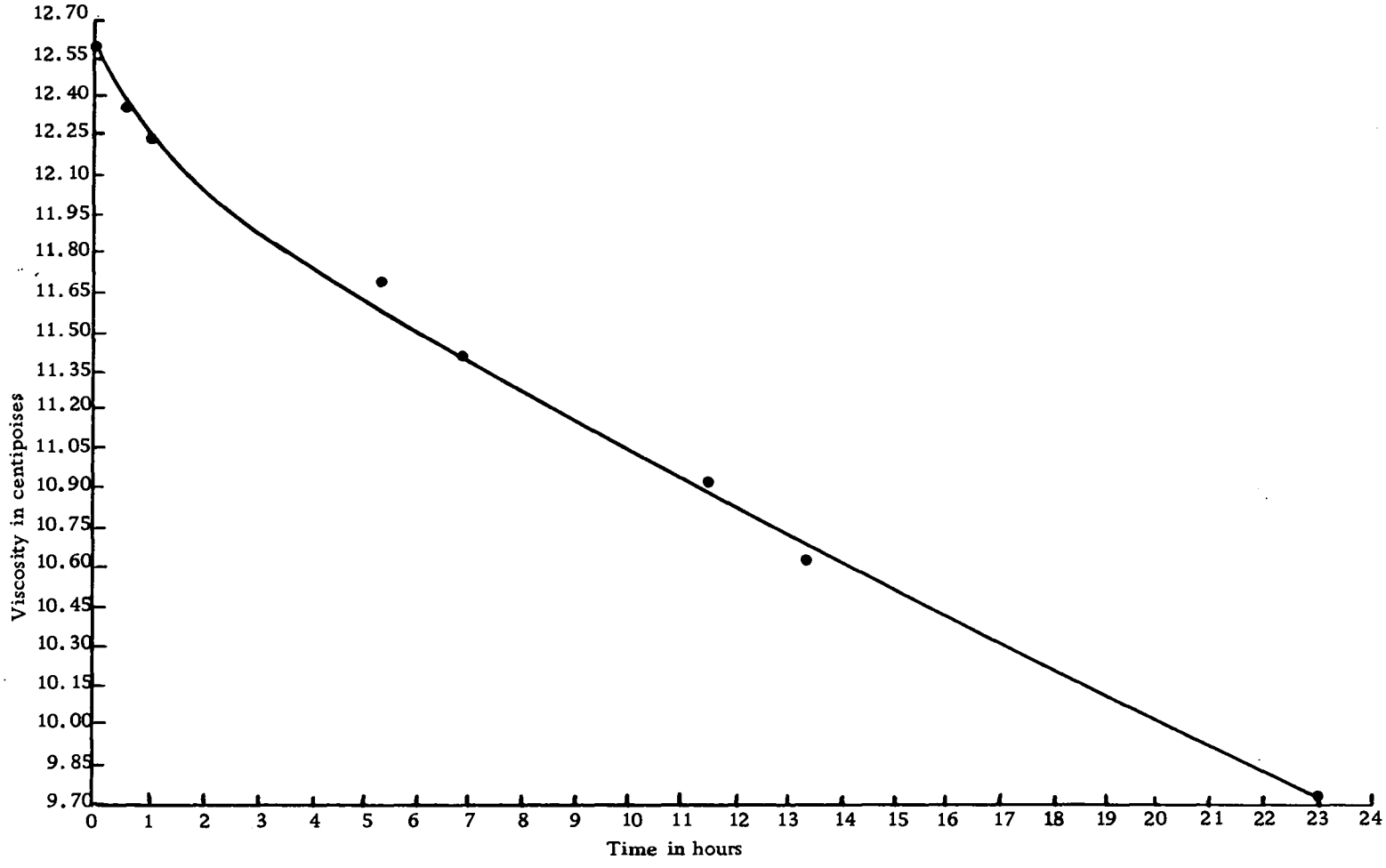


Figure 8. Viscosity of a 1.0 percent citrus pectin solution as a function of the time of action of strawberry proteins.

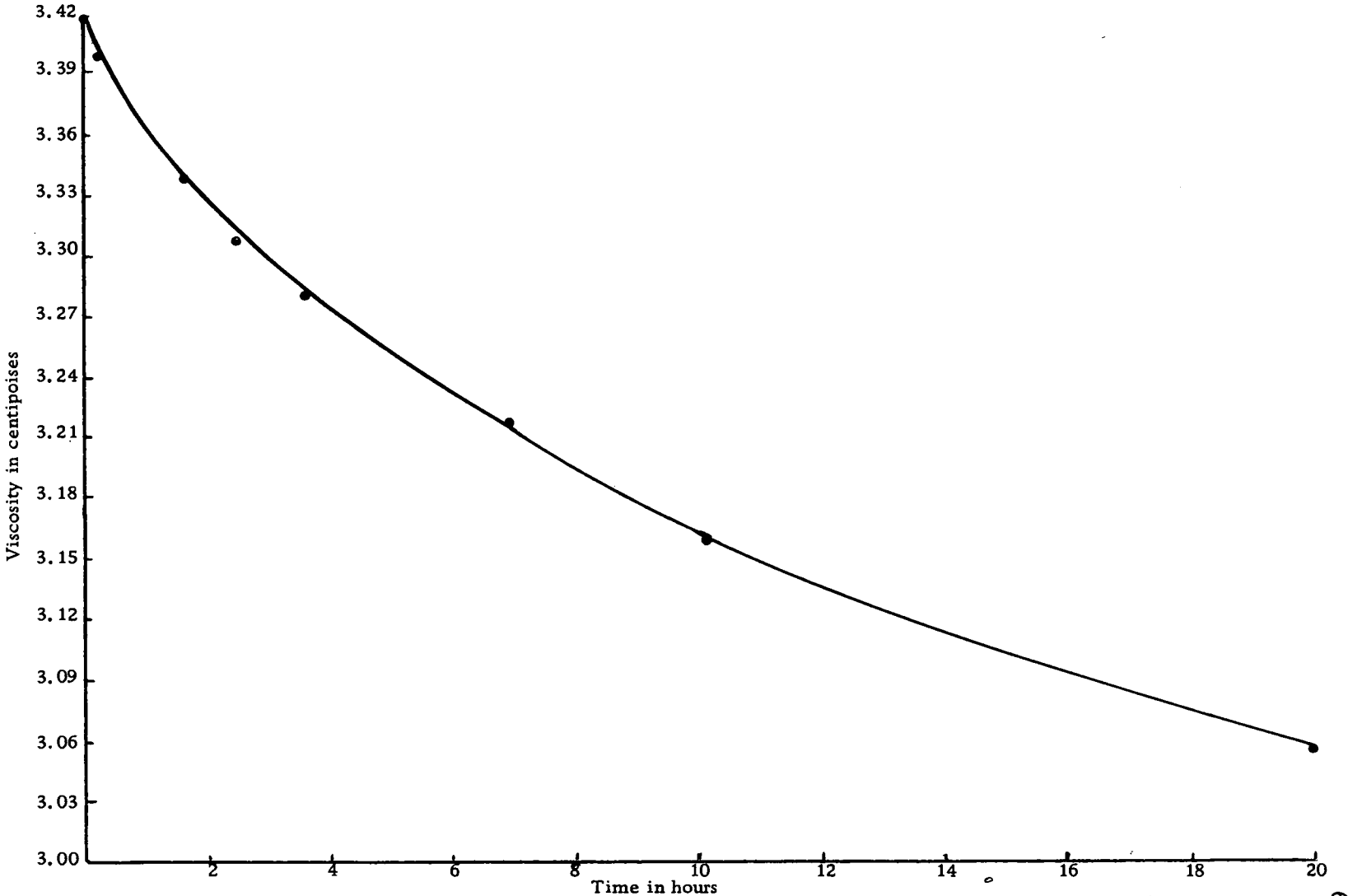


Figure 9. Course of action of strawberry pectins on water soluble fraction of strawberry AIS.

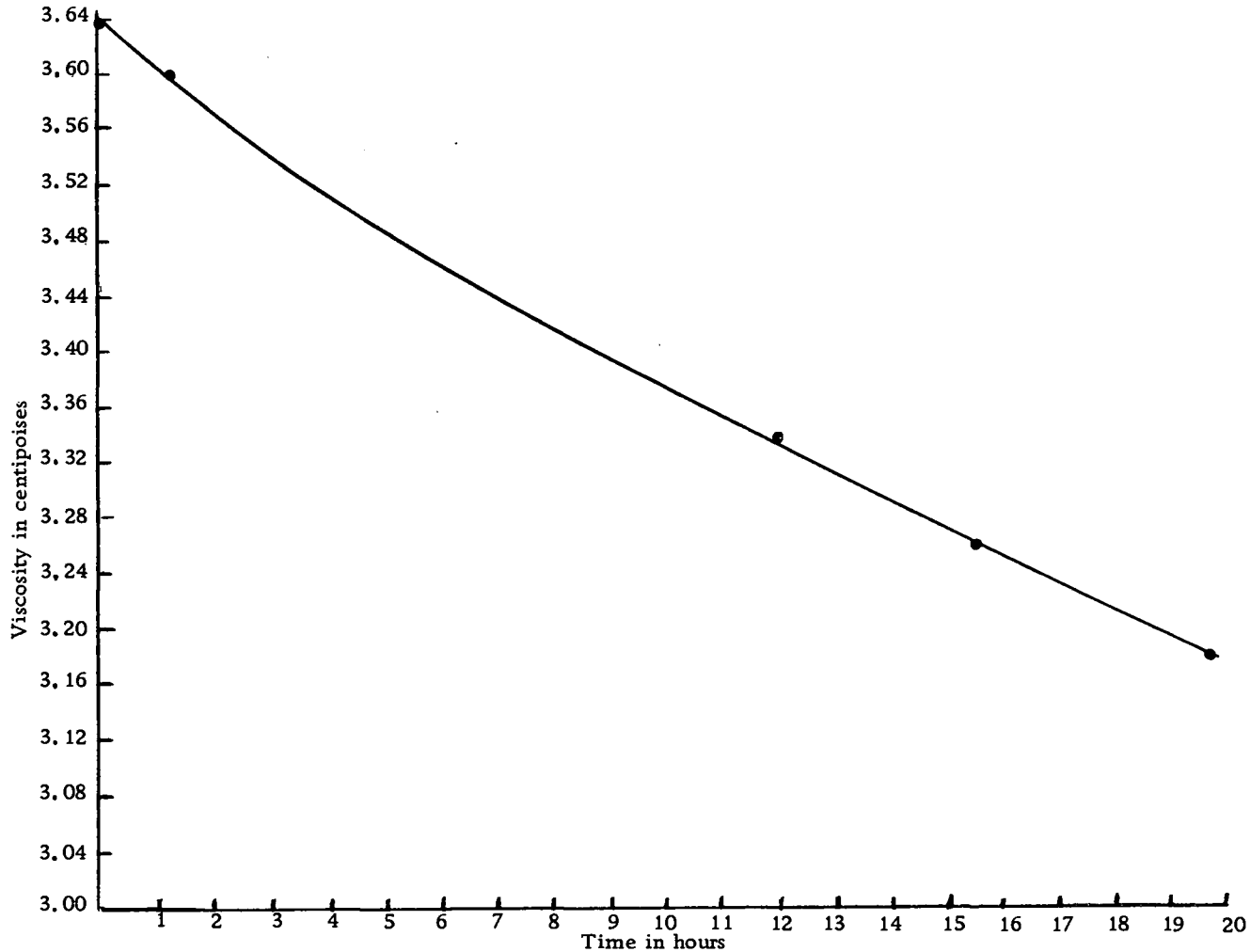


Figure 10. Effect of strawberry proteins on hexametaphosphate soluble fraction of strawberry alcohol insoluble fractions.

instead of the protein solution, was added. Toluene, at a level of one percent, was used as bacteriostatic agent in all the samples.

The results in Table 4 show that the strawberry proteins exhibited a catalytic action on the decrease in the viscosity of the solutions of various pectic substances. The decrease in the viscosity of the blank indicated that hydrolysis existed even in the absence of the protein solution. The loss of viscosity in the nonprotein samples (blanks) is similar for three of the substrates, but is noticeable less in the case of the citrus pectins. It might be postulated from this data that the presence of the methoxyl groups in citrus pectins blocks the acid hydrolysis of the methylated molecules.

The degree of methylation of the citrus pectins had no effect on the pectinolytic activity since the sodium pectate and the high methoxyl citrus pectins showed the same reaction rate. A difference in reaction rate occurred between the strawberry and citrus pectins. This fact suggests that the strawberry pectins may differ chemically from the citrus pectins.

A catalytic agent, probably an enzyme, exists in the strawberry based on the data in Table 4. The action of this agent contributed to the breakdown of pectic substances from the middle lamella of the strawberry which would effect the texture of the strawberry.

In the following section, the effect of pH on the activity of the strawberry pectinolytic enzyme was studied. The purpose was to find the optimum pH of this enzyme and to compare this pH with the normal pH of the fruit.

Optimum pH of the pectinolytic activity: The pH optimum of the pectinolytic activity of the strawberry proteins was studied in a series of measurements of the percent decrease in the viscosity of 1.0 percent sodium pectate solutions in which pH was the variable. The temperature was 30°C (86°F), the protein added corresponded to 0.025 grams of acetone powder and toluene was added as a bacteriostatic agent. Distilled water, instead of protein solution, was added to the blanks.

Table 5. Pectinolytic activity as a function of the pH after a 20 hour period at 30°C.

Means of 10 determinations

pH	Percent loss in viscosity			Coefficient of variation	Pectinolytic activity units
	Protein added	Blank	Difference		
3.00	27.8	12.0	15.8	1.5	780
4.00	27.0	9.0	18.0	1.2	920
4.50	26.8	7.0	19.8	1.3	1030
5.00	26.0	6.0	20.0	1.3	1040
5.50	25.5	5.5	20.0	1.1	1040
6.00	22.7	5.0	17.7	1.4	910
7.00	20.2	5.0	15.2	1.2	770

The citrate buffer was prepared as described in the Materials and Methods and adjusted to the desired pH.

A pH between 4.5 and 5.5 appeared to be the optimum for the strawberry pectinolytic enzyme. Since the pH of the strawberry is about 3.4, this enzyme is most active at a pH somewhat above the pH of the fruit.

A broad optimum pH range has been reported for other pectinolytic preparations (18, p. 122). Endopolygalacturonase found in the culture fluid of Saccharomyces fragilis had an optimum pH range of 4.0 to 4.8 (54).

Figure 11 shows the effect of the pH on the pectinolytic activity and Figure 12 shows the non-enzymic hydrolysis of the sodium pectate as a function of pH.

In the work that follows the effect of salt concentration on the pectinolytic activity of the strawberry proteins was studied. The concentration of salts is reported to have an influence on the activity of some pectic enzyme preparations (46, p. 370).

Effect of salt concentration on the pectinolytic activity: The effect of sodium chloride in concentrations of 0.25 M and 0.50 M was studied. The enzyme activity at different levels of salt concentration was tested at pH 5.0 and 30°C (86°F). The salt lowered the initial viscosities of the solutions but the percentage decrease in viscosity during storage was the same in the control sample to which salt was not added as in samples containing 0.25 M and 0.50 M

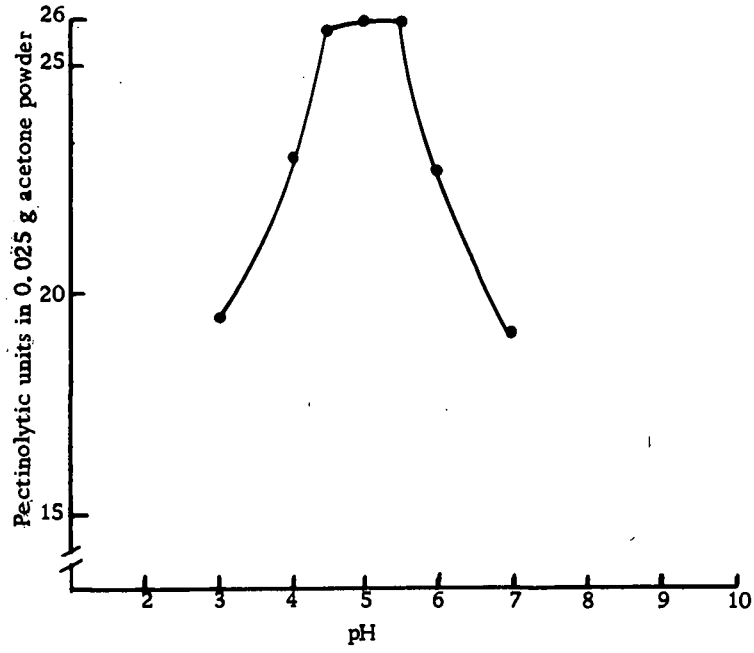


Figure 11. Effect of the pH on the activity of strawberry polygalacturonase.

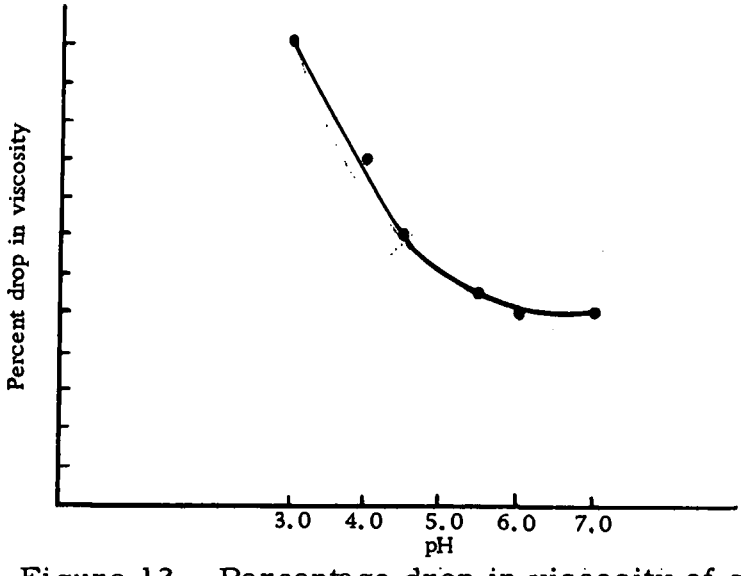


Figure 12. Percentage drop in viscosity of sodium pectate solutions at different pH and temperature 30°C.

sodium chloride. Sodium pectate solution was used as substrate. The results are given in Table 6.

Table 6. Effect of sodium chloride concentration on the action of strawberry enzyme on 1.0 percent sodium pectate solution at pH 5.0 at 30°C.

	Means of three determinations	
Salt concentration (moles/lit)	Initial viscosity (centipoises)	Percentage loss in viscosity (20 hour period)
0	14.30	20
0.25	14.28	20
0.50	14.25	20

The effect of calcium ion at 0.01 M concentration was studied using a pectin substrate, since even in minute concentrations, calcium caused gelation in sodium pectate solutions.

No significant effect of Na or Ca cations on the activity of the strawberry pectinolytic enzyme was found. The activities are comparable with those in Table 4. If there is cation requirement, the buffer cation fulfilled it. The concentration of the sodium ion in the buffer was 0.02 M.

Heat inactivation of the pectinolytic activity: The heat stability of the strawberry pectinolytic enzyme is important from the technological standpoint. Strawberry acetone powder was extracted with distilled water, the proteins precipitated with ammonium sulfate and

the protein solution dialyzed. Citrate buffer at pH 5.0 was added to the solution and 5 ml were heated for 20 and 35 minutes at 100°C (212°F). The effect of the heated protein solutions on the viscosity of a one percent sodium pectate solution in citrate buffer, pH 5.0, at 30°C was tested. For the control, buffer instead of the protein solution was added. The percent change is based on a 44 hour period. The protein added to each sample corresponded to 0.025 g of acetone powder. The results are given in Table 7.

Table 7. Effect of boiling on the pectinolytic activity of the strawberry proteins.

Means of 10 determinations

Substrate	Percent loss in viscosity	Loss due to catalytic action	Coefficient of variation
Substrate + protein solution	49	39	0.6
Substrate + enzyme boiled for 20 minutes	15	5	4.0
Substrate + enzyme boiled for 35 minutes	11	1	7.0
Substrate + water	10		3.6

Figure 13 gives the TDR curve for the pectinolytic activity of the strawberry proteins in citrate buffer, pH 5.0. Heating at 212°F for longer than 35 minutes is required for complete inactivation of the pectinolytic activity of the strawberry proteins. The heat treatment normally given to strawberries, 18 minutes at 212° F in No. 2 cans and 60° syrup, is not sufficient to completely inactivate this pectinolytic enzyme.

The strawberry pectinolytic enzyme appeared to be more heat stable than other pectic enzyme preparations. Roboz et al. (84) reported that an endopolymethylgalacturonase preparation from Neurospora crassa was 50 percent inactivated at 40°C in one hour with complete inactivation occurring at 70°C, at pH 6. Ayres et al.

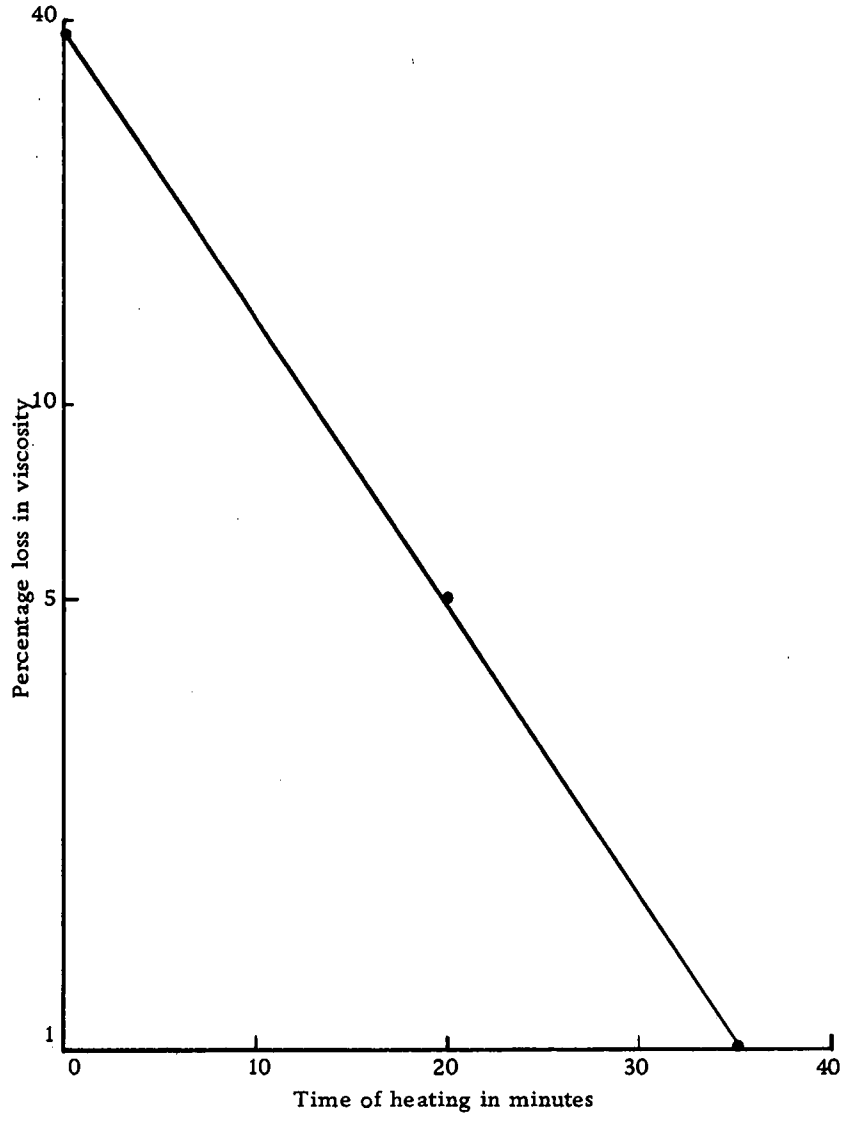


Figure 13. TDR curve for the pectinolytic activity of the strawberry proteins at pH 5.00 on sodium pectate

(5) observed destruction of the enzyme from Aspergillus foetidus at 50°C after 30 minutes at pH 5 and Brooks and Reid (14) found a pectinolytic enzyme from Aspergillus foetidus whose activity remained after heating at 60°C for 20 minutes.

The strawberry pectinolytic enzyme appeared to be quite heat stable being only partially inactivated when boiled for periods of time corresponding to the heat treatment period given to canned strawberries. The pectinolytic activity could play a part in the texture breakdown of canned strawberries.

After establishing the pH optimum and the heat stability of the pectinolytic activity of the strawberry protein, the effect of methylation of the substrate on the activity was investigated.

Pectinolytic activity of strawberry protein on demethylated calgon-soluble fraction of strawberry A. I. S.: The data in Table 4 suggested that there is no substrate preference between citrus sodium pectate and citrus pectin. Based on this observation it could be concluded that the degree of substrate methylation has no influence upon the pectinolytic activity. For further substantiation, a solution of calgon-soluble strawberry pectin was demethylated with 0.25 N sodium hydroxide by the procedure used for the determination of MeO content. The pH of the solution was brought back to pH 5.0 and citrate buffer at pH 5.0 was added.

The final concentration of the substrate was one percent and the enzyme added corresponded to 0.025 g of acetone powder. The

change in viscosity for a period of 20 hours was measured at 30°C (86°F). The means of 10 determinations are given in Table 8. Figure 14 gives the course of the action of the strawberry proteins on the demethylated pectin.

Table 8. Percentage viscosity loss of demethylated calgon-soluble strawberry pectins.

	Means of 10 determinations		
Substrate	Percent loss	Difference	Coefficient of variation
Demethylated calgon fraction	14.7	7.7	2
Calgon fraction	15.9	8.9	2
Blank	7		

The percentage viscosity loss of the demethylated pectin was slightly lower than in the case of the untreated sample. The difference could be attributed to a change in the substrate because of the alkaline degradation of the chain and not to the different degree of esterification of the substrate. The difference in the initial viscosity of the solutions in Figures 10 and 14 is due to the different salt concentrations and to partial degradation of the chain of the pectic substances. The effect of the salt concentration on the viscosity is small. The degree of esterification did not seem to have any significant influence on the pectinolytic activity of the strawberry proteins.

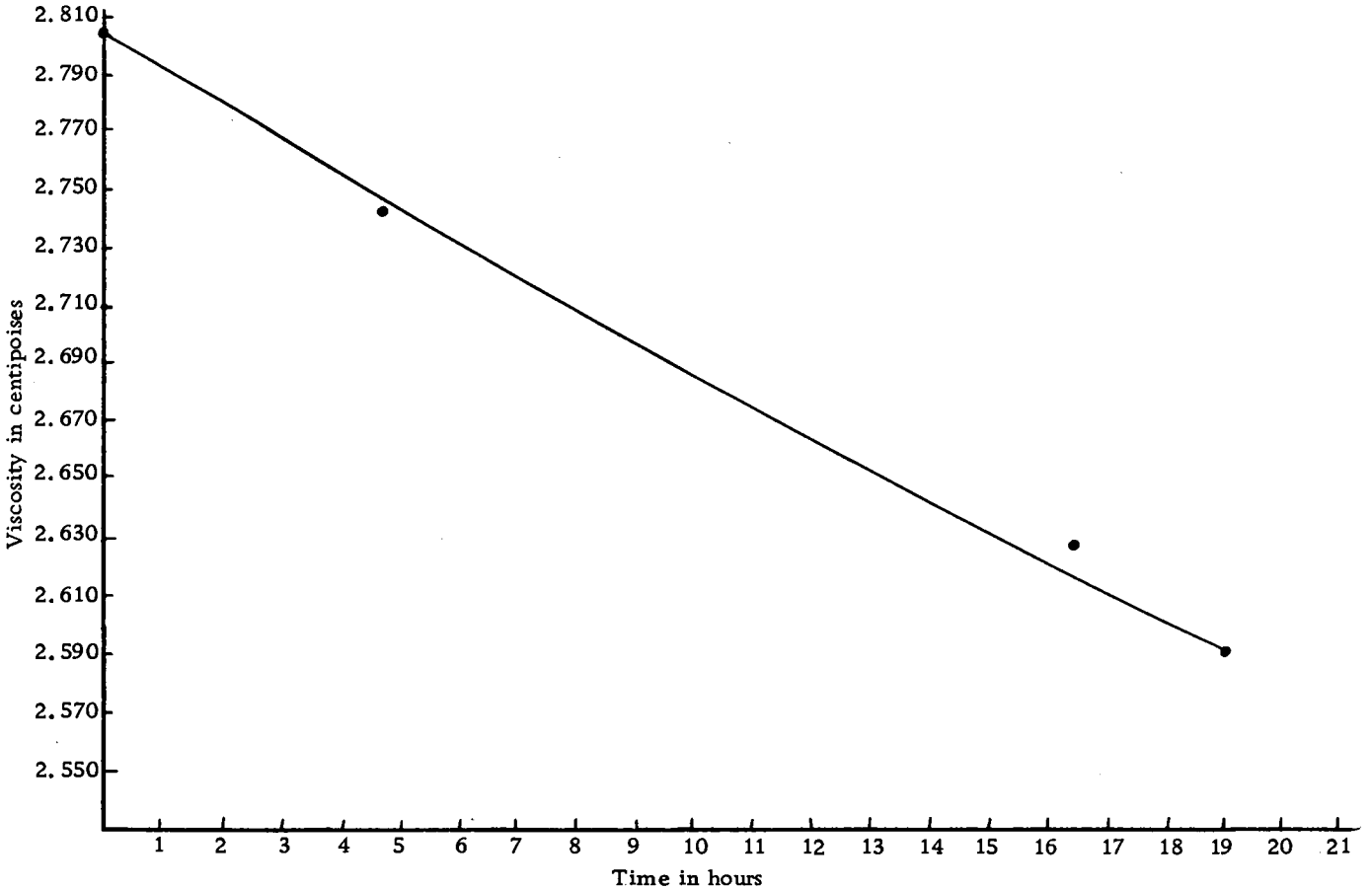


Figure 14. Course of action of straw protein on demethylated calgon-soluble fraction of A. I. S.

The catalytic effect of the strawberry proteins on the decrease in viscosity of water-soluble and calgon-soluble fraction of the strawberry A. I. S. was reported in previous sections of this study. Insoluble pectic substances, protopectin, are important constituents of the primary cell wall and the catalytic effect of the strawberry proteins on the rate of protopectin hydrolysis was studied.

Effect of the strawberry proteins on the hydrolysis of the insoluble residue of the strawberry A. I. S.: To study this effect, 8 g of the insoluble residue were dispersed in 300 ml of citrate buffer at pH 5. 0. To 150 ml of the dispersion, protein solution equivalent to 0. 4 g of acetone powder was added. The remainder of the suspension was used as a blank after addition of distilled water instead of protein solution. Toluene, at the level of one percent, was added as a bacteriostatic agent. The samples were kept at 36°C (97°F) under continuous stirring. Initially, and at timed intervals, samples were taken, filtered and analyzed for carbohydrate content by the phenol-sulfuric acid colorimetric method. The results are tabulated in Table 9.

Data in Table 9 indicated that hydrolysis of either the protopectin or the cellulose or both was accelerated by the addition of the strawberry protein solution.

Table 9. Effect of strawberry proteins on the insoluble residue of the A. I. S.

Means of five determinations

Time hours	Soluble sugars expressed as glucose in µg/ml			
	Protein added	Blank	Difference	Coefficient of variation
6. 25	2, 000	1, 850	150	18
19. 00	2, 250	1, 900	350	16
25. 50	2, 350	1, 900	450	14
31. 50	2, 600	1, 950	650	13
42. 50	2, 750	1, 950	800	12

The protopectin was removed from the insoluble residue by hydrolysis with two percent sodium hydroxide. The residue remaining after the alkaline treatment was washed with distilled water and the pH was adjusted with citric acid to pH 5. 0. The dispersion was suspended in citrate buffer pH 5. 0. Purified proteins from the strawberry acetone powder corresponding to 0. 4 g of acetone powder were added to the sample and distilled water to the blank. Samples and blanks were incubated at 36°C (97°F) under continuous stirring, with toluene added as a preservative. The soluble sugars were determined by the phenol-sulfuric acid method. Results are given in Table 10.

It could be concluded that the strawberry proteins did not demonstrate any catalytic effect on the hydrolysis of the protopectin-free insoluble fraction of the A. I. S. The increase in soluble sugars shown in Table 9 must be attributed, therefore, to hydrolysis of the

Table 10. The effect of strawberry proteins on the protopectin-free insoluble residue of the A. I. S.

Means of five determinations

Time in hours	Soluble sugars expressed in µg/ml as glucose		
	Protein added	Blank	Difference
0	150	100	50
10	180	120	60
22	200	140	60

protopectin. The strawberry proteins demonstrated a protopectinase activity according to the data of the Tables 9 and 10. The strawberry proteins appear to be able to catalyze the hydrolysis of the protopectin of the cell wall of the strawberry and thus promote softening of the fruit cells.

Action of Commercial Pectic Enzymes on the Pectic Substance of Strawberries

The data in Table 4 indicated that the percent loss in viscosity of the calgon-soluble fraction of the strawberry A. I. S. was more pronounced than the same loss in the water-soluble fraction of the A. I. S. The data in Table 1 indicated that the uronide content of the water-soluble fraction was higher than that of the calgon fraction and because of this observation a more extended viscosity loss in the water-soluble fraction was expected. To investigate the cause of this discrepancy, commercial pectic enzymes were tested against the pectin substances extracted from the strawberry A. I. S.

The commercial enzymes used were Pectinol-10 obtained from the Rohm and Haas Co. and Pectinase-D which was obtained from Miles Chemical Corporation. These two commercial enzymes are mixtures of pectic enzymes and have been used for the isolation of pure pectic enzymes (46, p. 376).

Table 11 tabulates the percent viscosity decrease in one percent solution of the water-soluble fraction or the calgon-soluble fraction of the strawberry alcohol insoluble solids in citrate buffer, pH 5.0. The temperature of the incubation was 30°C, the amount of the enzyme used in each determination corresponded to 0.005 g of commercial enzyme and the incubation period, 12 hours.

Table 11. Percentage loss in viscosity of water and calgon soluble strawberry pectins during the action of commercial enzymes for a 12 hour period.

Means of three determinations

Enzyme	Water soluble	Calgon soluble
Pectinase-D	44	41.3
Pectinol-10	50	42.2

The data in Table 11 indicated that the commercial pectic enzymes, which are mixtures of different pectic enzymes, gave a greater percent loss in viscosity in the case of the water-soluble pectin than in the case of the calgon-soluble pectin. The difference could be attributed to the higher uronide content of the water-soluble pectin, as indicated in Table 1.

Pectinesterase Activity in Northwest Strawberry

Pectinesterase activity exists in most plant tissues (74). The activity of this enzyme in the Northwest strawberry was studied in this work. Preliminary tests had shown the presence of pectinesterase activity in sodium chloride extracts of the tissue and in the acetone powder. After the establishment of the presence of pectinesterase in the Northwest strawberry, the optimum pH of this enzyme was determined.

pH Optimum: Continuous titrations at five different pH values were carried out to establish the pH optimum of this enzyme. Table 12 gives the means of 10 determinations of the pectinesterase activity at 30°C and decreasing pH values. A water extract of the acetone powder was used as the enzyme preparation.

Table 12. Pectinesterase activity at different pH value.

	Means of 10 determinations		
pH	Per gram of Acetone powder	Per 100 grams of tissue	Coefficient of variation
7.5	0.14	0.22	3
6.2	0.125	0.20	3
5.8	0.12	0.19	4
4.5	0.11	0.17	3
3.6	0.095	0.15	4

The substrate for the pectinesterase determination was a one percent, high methoxyl, citrus pectin solution in 0.15 M sodium chloride.

The results in Table 11 indicated that the optimum pH for the determination of the strawberry pectinesterase was 7.5. The activity of the enzyme decreased as the pH decreased. At pH values above 8 alkaline hydrolysis of the methyl group takes place and the results of the enzymic activity determinations are doubtful above pH 7.5.

After establishing the pH optimum, a quantitative determination of the pectinesterase activity was carried out.

Amount of pectinesterase activity: A series of 10 determinations of the pectinesterase activity of the acetone powder were done using a citrus pectin substrate. The pectinesterase activity in strawberry was found to be 0.22 units per 100 grams of tissue.

Table 13 gives the results of six determinations of the pectinesterase activity using three different ways of preparing the enzyme solution. In the first method, a distilled water extract of the acetone powder was used as the enzyme source. In the second, an extract of the strawberry tissue with sodium chloride solution and in the third method the dialyzed solution of the ammonium sulfate precipitate of the distilled water extract of the strawberry acetone powder were used as enzyme solutions. The conditions of these determinations are described in the Materials and Methods section.

Table 13. Comparison of methods of extraction of the pectinesterase.

Means of 10 determinations

Method of preparation	Activity in meq per 100 g tissue	Coefficient of variation
Acetone powder	0.22	3
Direct extraction with sodium chloride	0.18	4
Acetone powder-precipitation-dialysis	0.13	4

The pectinesterase activity in Northwest strawberry is low in comparison to the activity in tomato fruit. In tomato fruit there are 6-8 units per 100 g of fresh tissue.

The results in Tables 4 and 8 indicated that the activity of the pectinolytic factor in strawberry may be slightly affected by the degree of methylation of the substrate. The low pectinesterase activity in strawberry might be a limiting factor on the action of the strawberry pectinolytic factor. Demethylation of the pectin does not affect the viscosity of the solution (46, p. 163) except in the case where calcium ions form salts with the liberated carboxyl groups.

Pectin Transeliminase Activity

The presence of pectin transeliminase in strawberry was checked by following the change in the absorption of the light at 235 mμ of a 0.5 percent citrus pectin solution in 0.1 M citrate-phosphate buffer, pH 5.2, after the addition of the same buffer extract of the acetone powder.

No pectin transeliminase activity in strawberry could be demonstrated by this method.

Cellulase Activity in Strawberry

Cellulose plays an important role in the texture of fruits and a breakdown of the cellulose cell wall would affect the texture of these products. For this reason the presence of cellulolytic activity in the strawberry proteins was investigated. The protein solution from the acetone powder was used as the enzyme solution. The presence of cellulolytic activity was determined by the decrease in the viscosity of a solution of a soluble cellulose derivative. A 1.0 percent carboxymethylcellulose-7MP solution in 0.017 M citrate buffer at pH 5.0 was used as the substrate. The temperature of the viscosity determination was 30°C (86°F). Table 14 tabulates the data obtained from the determination of the cellulolytic activity of the strawberry proteins. The amount of protein present in each sample corresponded to 0.05 g of acetone powder.

Table 14. Percentage loss in viscosity of a carboxymethyl cellulose solution for a 20 hour period at 30°C.

Means of 10 determinations

Incubation mixture	Percent loss in viscosity	Net percent loss	C of V*	Cellulolytic units
Substrate + enzyme	52	50	2	3,150 per 100 g of fresh tissue
Substrate + water	2			
Substrate + enzyme boiled for 10 minutes	2	0		

* Coefficient of variation.

There were more than 3,000 cellulolytic activity units per 100 g of Northwest strawberry. Results in Table 10 indicated that the strawberry protein solution did not demonstrate catalytic activity on the solubilization of the protopectin free insoluble residue of the strawberry A. I. S. which was considered to consist mostly of cellulose. Possibly the cellulolytic enzyme of strawberry requires a soluble cellulose derivative as a substrate. By analogy to the variety of pectinolytic enzymes, it could be concluded that enzymes exist which show a preference between cellulose and cellulose derivatives. In this study the presence in strawberry of a protein having the ability to hydrolyze the insoluble cellulose prepared from strawberry was not demonstrated, although an enzyme capable of acting upon soluble cellulose derivatives is present in the Northwest strawberry. Ten minutes heating at 212°F inactivated the enzyme.

Chromatography on DEAE-Cellulose of Strawberry Proteins

The purpose for the work that follows was the demonstration of a method for the chromatographic separation of strawberry proteins.

Fifty mls of citrate buffer, pH 5.0 at 1°C were added to 1.5 g of acetone powder and the dispersion was allowed to stand at room temperature for 30 minutes. The proteins of the extract were

precipitated with ammonium sulfate, dissolved in distilled water and dialyzed for 14 hours at 1°C. The dialyzed protein solution was placed directly on a DEAE-cellulose column, 18 x 1.8 cm in dimensions. Fractions of 10 ml each were collected using a LKB 3400 B RadiRac fraction collector at 4°C. A sodium chloride concentration gradient was used for the elution. The eluate was checked for pectinolytic and cellulolytic activity. Sodium pectate, pectin, calgon-soluble strawberry pectin and water-soluble strawberry pectin at one percent concentration in citrate buffer, pH 5.0, were used as substrates for the determination of the pectinolytic activity at 30°C (86°F). One ml of eluate was added to the substrate as the enzyme test solution. Blanks with 0.1 M sodium chloride solution instead of enzyme solution were used. Pectinolytic activity was found only in the tube No. 8, in which the concentration of the sodium chloride was 0.1 M. Results of the determination of the pectinolytic activity of the eluate in tube No. 8 are given in Table 15.

Table 15. The percentage decrease in viscosity of different substrates using eluate of tube No. 8 as enzyme solution for a 20 hour period.

Means of six determinations

Substrate	Percent loss in viscosity				Pectinolytic units per g of acetone powder
	Eluate	Blank	Difference	C of V*	
Na Pectate	65	6	59	2.7	960
Citrus Pectin	61	3	58	3.0	
Calgon-soluble pectin	23	7	16	5.0	
Water-soluble pectin	15	7	8	5.0	

* Coefficient of variation.

The loss in pectinolytic activity because of the chromatography was approximately 10 percent. By using DEAE-cellulose column and sodium chloride gradient elution, the pectinolytic activity of the strawberry proteins was not separated into more than the single component.

The decrease in viscosity of a 1.0 percent, carboxymethyl cellulose solution at pH 5.0 for a 20 hour period was used for the determination of the cellulolytic activity. One ml of the eluate was used as the enzyme test solution and one ml of 0.2 M sodium chloride solution was used in the blank. The cellulolytic activity appeared in the tube No. 16 at a salt concentration of 0.2 M.

The results are given in Table 16.

Table 16. The percentage decrease in viscosity of a carboxymethyl cellulose solution at 30°C in 20 hours.

Means of six determinations

Substrate	Percent loss in viscosity			Cellulolytic activity units
	Total	Net	C of V *	
CM-cellulose + eluate	73	71	5	2,400 per g of acetone powder
CM-cellulose + salt solution	2			

* Coefficient of Variation

There was a decrease in the cellulolytic activity of about 25 percent as a result of chromatographic separation. The cellulolytic enzyme was not separated into more than one component.

Enzymic Nature of the Strawberry Pectinolytic and Cellulolytic Factors

Data in Tables 4, 7, 9, 14, 15 and 16 showed that the strawberry proteins have a catalytic effect on the decrease in the viscosity of pectic and cellulose derivative solutions. Since the viscosity is a function of the molecular weight of the macromolecules in solution (99, p. 407) it was concluded that a strawberry protein possessed the ability to break down pectic molecules and that a pectinolytic enzyme was present in the Northwest strawberry. The presence of a cellulolytic enzyme was indicated by the same data. In the section that follows a characterization of the pectinolytic enzyme was attempted.

Endoenzyme: Figure 8, 10, 7 and 9 indicated that there is an initial rapid loss in the viscosity of the pectic solutions immediately after the addition of the enzyme preparation. The catalytic effect of the enzyme on the viscosity drop was demonstrated from the initial phase of the enzymic action and thus the enzyme was characterized as an endoenzyme.

Endopolymethylgalacturonase: The data in Tables 4 and 8 indicated that the strawberry pectinolytic enzyme did not show any significant preference for pectic substrates of different degrees of methylation since the rate of the reaction of the strawberry pectinolytic enzyme on the highly methylated citrus pectin was not different

from that of the sodium pectate solution. There was a small difference in the rate of action upon the calgon-soluble strawberry pectins and the same pectins demethylated with alkali. The difference in the rate of action upon these two substrates could be attributed to partial hydrolysis of the pectins during the alkali treatment.

The enzyme could be characterized on the basis of the above discussion as an endopolymethylgalacturonase, which possesses the ability to act upon both pectates and pectinates.

Protopectinase activity: The data in Table 9 indicated that this enzyme can catalyze the hydrolysis of insoluble protopectin. The protopectinase activity of this enzyme on the insoluble cell wall pectins of the fruit must have a significant effect upon the texture of the strawberry. Data in Table 7 indicate that over 35 minutes heating at 212°F are required for the inactivation of this enzyme. The heat treatment given to canned strawberries is not sufficient for the complete inactivation of the enzyme. Probably the enzyme contributes to the softening of canned strawberries during both the processing and the storage of the product.

The pectinolytic enzyme occurring in the Northwest strawberry is an endopolymethylgalacturonase, which is capable of hydrolyzing pectins, pectates and protopectin. The enzyme demonstrated a pH optimum between 4.5 and 5.5 and was inactivated after boiling

for 35 minutes. Whether the enzyme is synthesized by the plant or is produced by microorganisms associated with the berry is not known.

SUMMARY AND CONCLUSIONS

The objective of this thesis was the development of a theory to explain the textural changes in fresh and processed strawberries. The presence of pectinolytic enzymes and the strawberry substrates upon which they act were studied. The following conclusions were made.

1. An endopolymethylgalacturonase exists in Northwest strawberries which catalyzes the hydrolysis of pectins, pectates and protopectins.

2. The strawberry endopolymethylgalacturonase indicated a maximum activity at pH values between 4.5 and 5.5.

3. The degree of methylation of the substrate did not appear to have an influence upon the activity of the enzyme. This enzyme demonstrated the same rate of action upon Na pectate and citrus pectin.

4. Sodium chloride solutions at concentrations up to 0.50 M and calcium ion at concentration 0.01 M did not show any effect on the activity of the strawberry endopolymethylgalacturonase in citrate buffer at pH 5.0.

5. The strawberry endopolymethylgalacturonase was inactivated after heating at 212° F for approximately 35 minutes in citrate buffer at pH 5.0.

6. Non-enzymic hydrolysis of the strawberry pectic substances occurs and hydrolysis is more pronounced at lower pH values.

7. The pectinesterase activity in Northwest strawberry is low in comparison with tomato fruits. The optimum activity of strawberry P. E. occurred at pH 7.5.

8. While cellulase activity existed in Northwest strawberries, the strawberry cellulase did not hydrolyze the insoluble strawberry cellulose.

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