to Dr A. E. Flood, of East Malling Research Station, who made available to us details of an improved ferricyanide method for the estimation of sugars.

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The Influence of Tannins on the Degradation of Pectin by Pectinase Enzymes

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When a high proportion of the cell-wall polysaccharides of myrobalans was found to consist of polygalacturonic acid (Hathway & Seakins, 1958), it became important to establish whether the available pectinases effect the degradation of pectin and low-methoxy pectin in the presence of myrobalans gallo- and ellagi-tannins. The effect of gambir-tannin extractives which contain (+)-catechin and phlobatannin on the enzymic degradation of pectin and low-methoxy pectin was also investigated.

MATERIALS AND METHODS

Paper chromatography was effected in all-glass apparatus at a constant temperature of 25°. Chromatograms were dried at room temperature, unless otherwise stated.

Pectin (apple) 240-grade (British Drug Houses Ltd.) was purified by repeated extraction with aq. 70% (v/v) ethanol, and the residue was dried at 35° and contained 18.6% of OMe.

Low-methoxy pectin was prepared by treatment of 50 g. of the commercial pectin with a mixture of 188 ml. of aq. 95% (v/v) ethanol and 62 ml. of N-NaOH for 20 min., and the precipitate obtained was washed with a mixture of 100 ml. of 95% ethanol, 10 ml. of water and 5 ml. of

N-HCl, and dried at 35°. Low-methoxy pectin contained <0.5% of OMe.

Pectin from the myrobalans cell-wall contained 16.0% of OMe.

Commercial pectinase preparations used in this work include Pectozyme (Norman Evans and Rais Ltd., Stockport, Ches.), Pectinol-100D (Rohm and Haas Co., Philadelphia, Pa., U.S.A.), Pektinol klarlöslich doppelkonzentriert (Rohm und Haas A.-G., Darmstadt, Hessen, Germany). Since it was proposed to investigate the pectolytic action of pectic enzymes of fungal origin in the presence of hydrolysable tannins, it was necessary to determine whether they exhibit tannase activity, as tannase can be obtained from cultures of such fungi as *Aspergillus niger* which have been grown in the presence of myrobalans-tannin extractives (Freudenberg, 1921; Freudenberg & Vollbrecht, 1921). A 5% (w/v) soln. of myrobalans-tannin extractives was accordingly digested with a large excess of the three enzyme preparations at 25° for 1 week, when two-dimensional paper chromatograms of myrobalans extractives before and after enzyme treatment were compared (Hathway, 1956). The presence of such hydrolysable tannins as chebulagic and chebulinic acids and corilagin in approximately the same proportions on both sets of chromatograms suggests that these enzyme preparations are devoid of tannase activity. Known substrates of tannase include chebulinic acid and gallotannin.

Myrobalans-tannin extractives refer to the methanol extract of the ground meal and gambir-tannin extractives refer to the commercial extract derived from the harvested leaves of *Uncaria gambir* Roxb. Gallotannin was obtained free from *m*-digallic and gallic acid contamination by the continuous ethyl acetate extraction of a solution of tannic acid (British Drug Houses Ltd.) in aq. NaHCO_3 soln. in a Schacherl apparatus, followed by evaporation of the solvent (Paniker & Stiasny, 1911).

Action of pectinase on pectin. A 1% (w/v) soln. of the pectins was prepared. Since the salt concentration produced by the addition of adequate buffer to maintain a constant pH would prevent the resolution of the oligogalacturonic acids and D-galacturonic acid by paper chromatography, the pH was adjusted to 6.0 by the addition of small volumes of N-HCl or N-NaOH . During pectolysis, the pH of the pectin solutions was maintained by the addition of small volumes of N-NaOH to neutralize the additional free carboxyl groups which were liberated. Experiments with tannin extractives were conducted at pH 3.0, and such pectin solutions were centrifuged to remove traces of insoluble material. Pectin solutions (1%) were also employed for viscometry.

Measurement of viscosity. The progress of the early stages of pectolytic action was followed by measuring the change in rate of flow of pectin solutions through a no. 2 Ostwald-type viscometer, B.S. 188:1937 (J. W. Towers and Co. Ltd.). Enzyme digests and viscometric measurements were conducted at $25 \pm 0.05^\circ$. In carrying out experiments which emphasized differences in pectolytic activity, due to different enzymes or to the inactivation of enzyme by tannin, such factors as concentration and pH were kept constant in the comparable situations.

Chromatography of D-galacturonic acid and the oligogalacturonic acids

Samples ($4 \times 5 \mu\text{l.}$) were withdrawn from digests during the course of pectolysis of low-methoxy pectin, and applied to appropriate positions on the starting line of a Whatman no. 1 filter paper, 57 cm. in length. This line of spots formed a time sequence from the beginning to the end of the reaction. Single-way chromatography was effected by the descending method, with water-saturated isobutyric acid (Jermyn & Tomkins, 1950) as solvent system. Papers were irrigated for 40 hr. or 60 hr., and dried (1 hr.) in an air current at $60-70^\circ$. Marker spots ($4 \times 5 \mu\text{l.}$) of 0.4% (w/v) solutions of authentic di-, tri- and tetra-galacturonic acid and D-galacturonic acid respectively were used. Treatment with a $\text{AgNO}_3\text{-NaOH}$ spraying reagent (Trevelyan, Procter & Harrison, 1950), followed by fixation in aq. 4% $\text{Na}_2\text{S}_2\text{O}_3$, was used to detect the acids, which gave intense black spots, as they possess 1:2-glycol groups (Hathway, 1956). Paper chromatography of those digests which contained tannin extractives, however, was effected with ethyl acetate-acetic acid-water (3:3:1, by vol.) mixture as solvent system. The papers were irrigated for 40 hr., and the ultimate product of reaction, D-galacturonic acid, was exposed as a red spot by means of the *p*-anisidine phosphate reagent. Duplicate experiments were set up for following the course of pectin hydrolysis. The first experiment was used for the detection of oligogalacturonic acids and D-galacturonic acid. In the second experiment, portions were adjusted to pH 9.0 and allowed to stand for 15 min., after which Na^+ ion was removed by

Zeo-Karb 225 cation-exchange resin in the H^+ form, and a similar line of spots was made on a second chromatogram.

Gallotannin-pectinase complex. A soln. of Pektinol klarlöslich doppelkonzentriert (0.16 g.) in water (50 ml.) was treated with excess of a 20% solution of gallotannin and centrifuged. The sediment was emulsified with water and separated in the centrifuge. The change in rate of flow of a 1% pectin soln. of pH 6.0 was measured during pectolysis by the gallotannin-enzyme complex, the original enzyme preparation and the enzyme regenerated from another portion of the gallotannin-enzyme complex by treatment with aq. 80% (v/v) acetone.

RESULTS

The present work represents an investigation of the effect of tannins on the pectolytic action of pectinase enzymes. A qualitative exploration of the course of pectolysis by means of paper chromatography showed that with all three enzyme preparations tested, and with low-methoxy pectin, a series of up to ten spots appeared on the developed chromatogram (Fig. 1). The fastest-moving spot, D-galacturonic acid, appeared simultaneously with the slower-moving ones, and it increased steadily in size and intensity until the end of the reaction, when it was the sole surviving spot. A typical pectolysis was complete within 3 or 4 days. The slower-moving spots waxed in intensity and size, and then waned and disappeared in the course of hydrolysis. Pectolysis of the selected substrate followed the same pattern in all three cases, and these enzyme preparations contrasted with Brooks & Reid's (1955) (D)-type enzyme and Schubert's (1952*a, b*) α -polygalacturonase, which gave a mixture of di- and tri-galacturonic acid and D-galacturonic acid. Parallel gelling tests with ethanol and calcium salts, however, showed that the colloidal properties disappeared more rapidly with Pektinol-100D than with Pektinol klarlöslich doppelkonzentriert. The more crude Pectozyme preparation was used only in the exploratory stages of the work.

Ashby, Brooks & Reid (1955) have recently isolated di-, tri- and tetra-galacturonic acid from enzyme hydrolysates, and the size and structure of these oligogalacturonic acids have been established (Jones & Reid, 1954, 1955). Di-, tri- and tetra-galacturonic acid have been used as markers in the present work, and they correspond to the second, third and fourth fastest-moving spots respectively. The regularity of the successive R_F values of galacturonic acid, di-, tri- and tetra-galacturonic acid, and of the remaining oligogalacturonic acids, suggests a series of increasing molecular complexity. When the R_M values [$\log (1/R_F - 1)$] of this series were calculated (Bate-Smith & Westall, 1950) and plotted against the size of polymer a linear relationship resulted

(Fig. 2). Hence there is a strong supposition that the spots exposed by development of the chromatograms may be identified as a series of oligogalacturonic acids up to the decamer.

A similar investigation of the pectolytic action of these enzymes on substrates of apple and myrobalans pectin, however, showed that galacturonic acid gradually accumulated during the reaction, and no oligogalacturonic acids were detected.

Further, when portions removed from these enzyme digests at intervals were treated with alkali before paper chromatography of the cation-free solutions, the developed chromatograms again showed an absence of polyuronide intermediates, and a gradual accumulation of galacturonic acid. The course of pectolysis of these pectins therefore differs profoundly from that of low-methoxy pectin.

Fig. 1. Sequence of low-galacturonic acid polymers and D-galacturonic acid obtained when a 1% solution of low-methoxy pectin, pH 6.0, is acted upon by Pectinol-100 D. This figure has been prepared from a chromatogram run at 25° for 40 hr. on Whatman no. 1 paper, in water-saturated isobutyric acid. For details see Methods. ⊖ Represents the spot of maximum intensity.

The progress of the initial stages of hydrolysis may be followed by measuring the change in time of flow of pectin solutions through an Ostwald-type viscometer tube. Such measurement, which is a function of viscosity, is dependent on the amount of unchanged pectin present. A comparison of the pectolytic action of Pectinol-100D and Pektinol klarlöslich doppelkonzentriert by these means is given in Fig. 3, for apple-pectin substrate at pH 6.0. The viscosity of the pectin solution falls rapidly until it approaches the value for water, when approximately 10% scission has occurred.

The presence of myrobalans tannins exerts an immediate inactivating influence on the pectolytic action of these enzyme preparations when a low-methoxy pectin solution of pH 3.0 is used as substrate (Fig. 4). When apple or myrobalans pectin is employed as substrate, a marked increase in viscosity is associated with the interaction between pectin and tannin, and in the course of hydrolysis the presence of myrobalans tannins exerts only a slight retarding influence on the pectolytic action. The rate of flow of the pectin solution falls steeply (Fig. 4). With pectin and low-methoxy pectin, pectolysis does, however, go to completion in the presence of myrobalans tannins. The presence of

Fig. 3. Change in flow-rate of pectin solutions, produced by pectolytic action. The reaction flask contained a solution of 1% apple pectin, pH 6.0. O, With 0.04% Pectinol-100D; Δ , with 0.16% Pektinol klarlöslich doppelkonzentriert. The ordinate refers to the time of flow through an Ostwald-type viscometer (for details see Methods).

Fig. 2. Change in R_M value with size of galacturonic acid polymer. Degree of polymerization is plotted against the distance travelled by the corresponding spot (O), and solvent front, and against the R_M value (●).

Fig. 4. Effect of myrobalans and gambir tannins on the change in flow-rate of pectin solutions brought about by pectolytic action. The reaction flask contained a solution of 1% low-methoxy pectin and 0.04% of Pectinol-100D, pH 3.0. O, Without tannin; ●, with 5% myrobalans-tannin extractives; ■, with 5% gambir-tannin extractives. A solution of 1% pectin and 0.04% of enzyme was used under similar conditions. □, Absence of tannin; Δ , presence of 5% myrobalans-tannin extractives; $\blacktriangle$, 5% gambir-tannin extractives. The ordinate refers to the time of flow through an Ostwald-type viscometer (for details see Methods).

gambir tannin has no effect on the pectolysis of pectin and low-methoxy pectin.

When a solution of Pektinol klarlöslich doppelkonzentriert was precipitated by gallotannin, the resulting enzyme-tannin complex exhibited no initial pectolytic activity, but as the complex began to dissociate in a pectin solution, hydrolysis commenced (Fig. 5). The enzyme, regenerated from the enzyme-tannin complex by solvent precipitation, possessed a reasonable proportion of its original pectolytic activity. This experiment attempts to simulate the extreme case in which the presence of a big preponderance of gallotannin might be expected completely to impede pectolysis.

DISCUSSION

The evidence suggests that the spots, encountered in the chromatographic study of the pectolysis of low-methoxy pectin by the commercial enzyme preparations, may be identified as a series of oligogalacturonic acids up to the decamer. In this reaction, the action of two enzymes, endopolygalacturonase (endo-PG) and exopolygalacturonase (exo-PG) (Demain & Phaff, 1957) can be distinguished. Endo-PG attacks low-methoxy pectin in preference to pectin by a random mechanism of hydrolysis, which leads to the series of intermediate oligogalacturonic acids, and eventually to digalacturonic acid; exo-PG attacks low-methoxy pectin in preference to pectin by a terminal

mechanism of hydrolysis. Thus in the present reaction, the action of exo-PG accounts for the appearance of galacturonic acid simultaneously with the oligogalacturonic acids produced by endo-PG. Further, the accumulation of a large amount of galacturonic acid before the formation of the intermediate oligogalacturonic acids when the enzyme level is lowered is readily understood on the assumption that two enzymes are involved in this reaction. Exo-PG also functions in the terminal removal of galacturonic acid units from the intermediate oligogalacturonic acids, and in the final scission of digalacturonic acid. The viscometric measurements showed that gambir tannin has no effect on the pectolytic action of endo-PG and exo-PG, whereas the immediate inactivation of pectolytic action in the presence of myrobalans tannins suggests that they inhibit the action of endo-PG.

The absence of oligogalacturonic acids and their methyl esters from the hydrolytic products of pectin can only be interpreted by the action of exopolymethylgalacturonase (exo-PMG) which attacks pectin in preference to low-methoxy pectin by a terminal mechanism of hydrolysis (Demain & Phaff, 1957). Some exo-PG action may also be involved in the overall hydrolysis, depending on the degree of methylation of the pectin. The hydrolytic action of exo-PMG is not affected by the presence of gambir tannin, but is slightly inactivated by myrobalans tannins.

These results show that the commercial enzyme preparations, Pektinol-100D and Pektinol klarlöslich doppelkonzentriert, are mixtures of at least three enzymes, endo-PG, exo-PG and exo-PMG. The results imply that the enzyme preparations would function efficiently in such natural substrates as blackcurrant juice, which contains phenolics and phlobatannin similar to those found in the gambir extractives. The fact that pectolysis can take place in the presence of myrobalans tannins is consistent with the ripening of the fruit, and the retarding action exerted by these tannins on the action of the pectolytic enzymes explains why sound, dry myrobalans can often be collected from the forest floor.

SUMMARY

1. Pectolysis of low-methoxy pectin by commercial enzyme preparations leads to a series of oligogalacturonic acid intermediates up to the decamer, and eventually to D-galacturonic acid, whereas the enzymic hydrolysis of pectin affords D-galacturonic acid alone.

2. The encountered pectolytic actions of these enzyme preparations may be explained in terms of endopolygalacturonase (endo-PG), exopolygalacturonase (exo-PG) and exopolymethylgalacturon-

Fig. 5. Change in flow-rate of a 1% apple-pectin solution, pH 6.0, produced by the pectolytic action of a gallotannin-enzyme complex. O, Complex derived from a 0.16% solution of Pektinol klarlöslich doppelkonzentriert; □, the enzyme regenerated from the complex (for details see Methods); Δ, the similar pectolytic action of the original enzyme.

ase (exo-PMG). Endo-PG attacks low-methoxy pectin by a random mechanism of hydrolysis which leads to intermediate oligogalacturonic acids and eventually to digalacturonic acid, whereas exo-PG removes terminal galacturonic units from the substrate and its intermediate polyuronides, and cleaves digalacturonic acid. Exo-PMG attacks pectin by a terminal mechanism of hydrolysis.

3. Viscometric measurement shows that myrobalans tannins exert a powerful inactivation on the early stages of the pectolysis of low-methoxy pectin, but only a slight retarding action on the pectolysis of pectin.

4. The fact that these enzymic hydrolyses go to completion in the presence of myrobalans tannins is consistent with the ripening of the fruit.

5. Gambir tannin has no effect on the pectolytic actions investigated.

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Distribution of Ascorbic Acid in Normal and Leukaemic Human Blood

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It has been known for some time that the buffy layer of normal human blood contains a high concentration of ascorbic acid (Butler & Cushman, 1940, 1941). The distribution between lymphocytes, granulocytes and platelets has, however, remained obscure and forms the subject of this present paper. Whereas previous workers have quoted their results on a wet-weight basis only, the data presented here are based on the analysis of individual cells determined by direct counting.

Leukaemic blood has been found to contain low concentrations of ascorbic acid, both in the serum (Hagtvet, 1945) and plasma and in the buffy layer (Waldo & Zipf, 1955). In contrast, Butler & Cushman (1940) found higher amounts than normal in the buffy layer. We have included analyses of chronic leukaemic human blood in this work, in order to investigate these claims and also to provide data on the relatively pure suspensions of lymphocytes and granulocytes obtainable in leukaemic diseases.

METHODS

Collection of blood. Blood (30-90 ml.) was aspirated from an antecubital vein with sterile paraffined all-glass syringes and no. 18 B.D. steel needles. It was immediately transferred to siliconized glass centrifuge tubes (10 ml.) containing as anticoagulant one part of disodium ethylenediaminetetra-acetate dihydrate (Sequestrene) soln. [1% (w/v) in 0.7% NaCl soln.] to nine parts of blood. The tubes were inverted several times to mix the contents.

Centrifuging. A horizontal refrigerated centrifuge, radius 14 cm., at a temperature of 4°, was used throughout.

Separation of the formed elements

Platelets. The whole blood was centrifuged at 800-1000 rev./min. for 10-15 min. The upper three-quarters of the supernatant platelet-rich plasma was carefully removed with siliconized Pasteur pipettes and transferred to polythene centrifuge tubes. (The blood remaining after removal of the platelet-rich plasma is called 'platelet-poor blood'.) After removal of a small measured quantity (about 0.01 ml.) for a direct platelet count (Barkhan, 1957), the measured volume of plasma was centrifuged at