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Cellulase from Leaves and Roots of Tobacco

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In the course of work on the enzymic breakdown of leaf fibre (Holden, Pirie & Tracey, 1950), it was found that leaf sap apparently has a weak cellulase activity. There are few reports of the existence of cellulase in the higher plants and all are concerned with its presence in germinating seeds or young seedlings (Newcombe, 1899; cf. Lüers & Volkamer, 1928; Grüss, 1928; Lüers & Malsch, 1929). Some preliminary observations on the presence of a cellulase in the tobacco plant and parts of some other plants are presented in this paper, because further work on the subject is not contemplated at the moment.

MATERIALS

Glasshouse-grown *Nicotiana tabacum* var. White Burley was used for much of the work, but parts of other plants listed later were also used. Tobacco plants were divided into leaves, stems and roots. The midribs were cut from the leaves, care being taken that as little as possible of the lamina was left attached to them. Roots were washed in running water until free from obvious contamination, and dried by pressing in cloth. The four batches of material were then minced separately in a domestic meat mincer and the sap squeezed out by hand through madapolam. The saps were centrifuged for 15 min. at about 1500 g and stored with CHCl_3 at 5°.

Snail digestive juice was got from the crops of starved snails as described by Bawden & Pirie (1946).

METHOD

Active plant extracts reduce the viscosity of a solution of sodium carboxymethylcellulose at pH 5. The rate of viscosity reduction is of the same order as that got with an

equal volume of a 1:100,000 dilution of *Helix* digestive juice (undiluted crop contents). This amount is too small for convenient measurement of its activity by methods other than viscosity reduction. Hyaluronidase activity is commonly measured (e.g. Madinaveitia & Quibell, 1940) by determining the time taken for half the viscosity increment due to the substrate to be lost. For accurate measurement by this method, it is necessary to know the viscosity at zero time and the viscosity at the completion of the action. Since relatively large volumes of sap must be used to get measurable viscosity reductions, and since the sap contains a substance or substances that react with the substrate, the zero time viscosity can only be measured by back extrapolation, which is not accurate owing to the rapid change in viscosity early in the reaction. The final viscosity cannot be measured since it would take a considerable time for the action to be completed, and during this time precipitation from the sap may occur. Consequently, the viscosity of the enzyme-substrate mixture was measured after 5 and 15 min. action and the rate of change of viscosity calculated. The value $(\log VI_5 - \log VI_{15})$ was used as a measure of the rate of change of viscosity (VI_5 and VI_{15} are the viscosity increments at 5 and 15 min.; the viscosity increment being taken as relative viscosity - 1). If the rate of viscosity reduction, produced by differing amounts of diluted *Helix* enzyme under the conditions specified below, is plotted against the amount of enzyme, a straight line is got when the amounts of *Helix* enzyme are in the range 0.002-0.01 μl . undiluted crop contents. With varying amounts of active tobacco sap the curve is smooth, and almost linear at the beginning. The variation from linearity may be due to variation in the amount of salts added in the sap and to variations in the amount of substrate combining with the material in the sap. It is possible, using such a curve, to make approximate estimations of the amount of enzyme present. The viscosity

increment of tobacco sap does not vary over a period of several hours in the absence of the substrate under the conditions used in the estimation. The very fine precipitate formed when lamina sap and substrate are mixed has a composition varying with the proportion in which sap and substrate are mixed. In conditions of substrate excess its N content is about 3% of the dry matter. The precipitate does not disappear during incubation, and it seems likely that it is formed by interaction of a protein fraction of the sap with the substrate. This precipitate was not observed when midrib sap or root sap was used, possibly owing to the lower protein-N content of these saps. A similar precipitate has been noticed when tobacco mosaic virus and sodium carboxymethylcellulose are mixed.

Measurement of activity. 1.2 ml. of a solution of sodium carboxymethylcellulose (dry matter approx. 10 g./l.) and 0.6 ml. of 0.2M-sodium acetate buffer (pH 5.0) are mixed in a 25 ml. beaker. Sap or sap dilution (1.2 ml.) is then added, mixed well with the contents of the beaker, and the time noted. 2.5 ml. of the mixture are pipetted immediately into an Ostwald viscometer supported in a water bath maintained at 25°. The time of flow is measured at times such that when half the flow time has elapsed 5 and 15 min. has passed since the time of mixing. From the flow times the relative viscosities and viscosity increments are calculated. The value of $(\log VI_5 - \log VI_{15})$ is used as a measure of activity, the unit of activity being defined as that activity which leads to a difference of 0.01 between $\log VI_5$ and $\log VI_{15}$ when acting on sodium carboxymethylcellulose of final concentration 4 g./l. The 'cellulase' unit is the amount of enzyme that produces this difference under the conditions described earlier. The amount of enzyme used is adjusted by dilution so that the total amount present is 2-3 units.

RESULTS

Properties of the enzyme

The optimum pH of the 'cellulase' was determined by using buffer solutions of differing pH's in the activity test. Either 0.2M-acetate or 0.2M-phosphate buffer was used, and pH measurements were made on the enzyme-substrate-buffer mixture after the completion of the viscosity determinations. Although the viscosity of the substrate shows a marked drop at low pH's there was little variation in the range used (pH 4-8). The optimum pH for viscosity reduction by tobacco cellulase was found in the region of pH 5.4.

Changes in the activity/ml. and activity/mg. protein N were measured after a number of treatments which led to the precipitation of part of the sap protein. Heating the sap for 3 min. at 55° followed by centrifuging led to the loss of about half the activity, with a decrease in activity/mg. protein N for both midrib and lamina sap. Activity/ml. can be increased by partial freezing of the sap followed by removal of the ice formed; activity/mg. protein N, however, is decreased. Adjustment of the sap to pH 4 resulted in the loss of all activity from the supernatant. An increase in activity/ml. was got by fractional precipitation with $(NH_4)_2SO_4$. About half the enzyme originally present can be recovered by extracting with water the precipitate produced by increasing the percentage saturation with $(NH_4)_2SO_4$ from 30 to 60. The saturated $(NH_4)_2SO_4$ solution used for precipitation was previously adjusted to a pH of about 5.5. No significant increase in activity/mg. protein N was observed in these preparations. Dialysis of sap results in the loss of about half

the activity/ml. in 2 days at 5° when compared with a sample stored the same time at the same temperature.

Variation of enzyme levels within the plant

Activity/ml. sap from different parts of the plant increased in the order lamina, midrib, stem, root. The activity of midrib and lamina saps decreased with increased age of the leaf. In four groups of midribs from the same set of plants the activity/ml. sap for the youngest group was 4.4 units, 3.4 and 2.7 units respectively for the two next older groups, and 1.4 units for the midribs from leaves that were beginning to lose their green colour. Similar decreases were found with lamina sap from leaves of increasing age, the activity being about three quarters that of the corresponding midrib saps. Midribs from which the vascular bundle has been removed before mincing give a sap of about the same activity/ml. as that from comparable whole midribs, but the activity/mg. protein N is higher. In the set of plants used to determine the effect of age of leaf on midrib-sap activity, the activity in units/ml. of the sap from the upper part of the stem was 2.5, and the activity of the root sap was about 12 units/ml. Since root and midrib sap contain less than a tenth of the amount of protein N that lamina sap does their activity/mg. protein N is much higher.

Cellulose as a substrate

If large amounts of active sap are used, it is possible to demonstrate the production of reducing groups following the action of the enzyme on cellulose in a finely divided form. The cellulose suspension (4 g./l.) was prepared by dissolving filter paper in syrupy phosphoric acid and precipitating by dilution with water. After dialysis the cellulose suspension still contained 1% P, equivalent to about 1 residue in 18 (5.5% of residues) being substituted with a phosphoric ester grouping. Root sap (3 ml.), having an activity of 8 units/ml., was incubated with 8 mg. of suspended cellulose at pH 5. After 22 hr. 0.51 mg. of reducing sugar had appeared in excess of blank values; incubation for a further 22 hr. only increased the amount to 0.62 mg. A solution of *Helix* enzyme containing in all 12 units of cellulase under the same conditions led to the formation of 0.28 mg. reducing sugar in 22 hr. and 0.60 mg. in 94 hr. Action on cellophan as substrate could not be unequivocally demonstrated with plant cellulase under these conditions, or with snail cellulase acting for 22 hr. However, after 94 hr. with snail cellulase demonstrable reducing sugar was liberated. It can be concluded that plant cellulase will split reprecipitated cellulose at about the same rate as that amount of *Helix* cellulase found equivalent by the viscosity method described above. Demonstration of action on cellophan by plant cellulase was not possible, since this material is less easily attacked, and the plant cellulase loses activity during long incubations.

Occurrence of the enzyme in other plants

Cellulase activity has been observed on single occasions in the sap of bean leaves (*Phaseolus vulgaris* L.), roots of beetroot and spinach beet (*Beta vulgaris* L., Vars.) and woody nightshade (*Solanum dulcamara* L.), flesh of vegetable marrow fruit (*Cucurbita pepo* L., Vars.) and in asparagus butts (*Asparagus officinalis* L.). The enzyme was not detected in the fruits of tomato (*Solanum lycopersicum* L.) or in rhubarb (*Rheum rhaponticum* L.) petioles; the sap of the first had a pH of 4.3, and that of the second 3.5. In all the saps in which activity was detected the pH was greater than 5.

DISCUSSION

The enzyme described has three properties in common with the cellulase of *Helix* and those of many moulds, it reduces the viscosity of a solution of sodium carboxymethylcellulose, it splits cellulose, and its optimal pH range is between 5 and 6. It has not been possible to show unequivocally that it is a cellulase, because it has not been got in a concentrated enough state to produce detectable breakdown of a completely unsubstituted cellulose. If it is, in fact, a cellulase, then it may be concerned in the breakdown of the fine cellulose fibrils of the primary cell wall of growing cells, some evidence for which has been supplied by Frey-Wyssling (1949). To explain occurrence of the enzyme in relatively higher concentrations in roots, where elongation and

renewal of root hairs is a continuous process, and in young leaves where growth is still going on, and the very low concentrations of enzyme got by extracting whole organs, it is reasonable to postulate a localization of the enzyme to the very small proportion of cells in which primary cell-wall modification is in process.

SUMMARY

1. An enzyme that can depolymerize sodium carboxymethylcellulose, and split reprecipitated cellulose has been found in tobacco plants.

2. The enzyme, which is similar in properties to animal and fungal cellulases, is present in very low concentrations. It is suggested that this may be explained by its localization in cells in which reconstruction of the thin primary cell wall is occurring as a concomitant of cell growth.

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A Colorimetric Method for the Determination of Pentoses in the Presence of Hexoses and Uronic Acids

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A method for the determination of pentoses in the presence of large amounts of hexoses and uronic acids has been developed for use in the examination of extracts of leaf fibre after enzyme treatments. Methods previously described are not satisfactory, owing to interference by hexoses or uronic acids or to lack of sensitivity. The survey by White & Green (1932) of colour tests for sugars and their derivatives in urine indicated that their qualitative 'aniline' test for pentoses might be developed. In this test, the sugar solution with an equal volume of glacial acetic acid and a few drops of aniline is heated to boiling, allowed to stand for 2 min., cooled and the colour extracted with chloroform. A quantitative modification of this test is now described, in which the interference by glucose and other sugars is reduced by allowing the reaction to take place at room temperature. It has the advantage that little manipulation or attention is required.

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EXPERIMENTAL

Method

100 ml. glacial acetic acid, 10 ml. 5% (w/v) aqueous oxalic acid, 24 ml. water and 16 ml. colourless aniline are mixed and stored in a dark-glass bottle. The reagent should be stored out of direct light and not be used more than a week after preparation. Reagent (6 ml.) is added from a burette to each of a series of test tubes (75 × 15 mm.) matched for use in a photoelectric absorptiometer (the Evans Electro-selenium Portable model was used). The solution to be tested (containing 10–80 µg. pentose) is added, together with enough water to bring the final volume to 8 ml. and the contents of the tube well mixed. At the same time a blank (2 ml. water + 6 ml. reagent) and two standards of xylose (10 and 50 µg.) are set up. If it is known that the pentose in the solution is not xylose, standards of that pentose are employed. Standard solutions of pentose containing 50 µg./ml. are conveniently made up in saturated benzoic acid solution. These standards do not deteriorate at room tem-