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Cellulase and Cell Differentiation in *Acer pseudoplatanus*

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Summary. Homogenates of differentiating xylem and phloem tissue have higher cellulase activities than cambial samples; the highest activity is always found in phloem. Callus tissue, in which no vascular differentiation occurs, contains only low cellulase activity. The results suggest that cellulase is involved in vascular differentiation. Different pH optima of cellulase activity were found: in cambium, xylem and phloem tissue, cellulase activity with an optimum at about pH 5.9 is predominantly membrane-bound; it is sedimentable at 100,000 g and releasable by Triton X-100. The same may be true of activity with an optimum at pH 5.3. Phloem tissue also contains a soluble, cytoplasmic cellulase of high activity at pH 7.1, and xylem tissue contains cytoplasmic cellulase with an optimum at pH 6.5. Low cellulase activity with a pH optimum similar to that of xylem homogenates was found in xylem sap. Cellulase activity in abscission zones increases greatly just before leaf abscission. Abscission zone cellulase has two pH optima, et 5.3 and 5.9; both activities are increased by Triton treatment of homogenates. The possible existence of several different cellulases forming part of a cellulase complex, and the rôle of the enzymes in hydrolysing wall material during cell differentiation are discussed.

Introduction

In higher plants the walls of certain cells are weakened or dissolved during development and differentiation, and it seems likely that cellulase plays a part in hydrolysing wall material during these processes. There is evidence (Sheldrake, 1969; Sheldrake and Moir, 1970) that cellulase is involved in the differentiation of articulated laticifers, in which end walls between cells disappear. In abscission zones a rise in cellulase activity is correlated with the weakening of cell walls which enables abscission to occur (Cracker and Abeles, 1969), and as tomato fruits ripen and cell walls gelatinize and weaken, cellulase activity increases (Hall, 1963). Two further examples of dissolution of cell walls are found in vascular differentiation. When xylem vessels differentiate, the end walls between cells disappear (cf. Sassen, 1965), and phloem sieve pores are made by the removal of wall material around plasmodesmata between differentiating sieve tubes (Northcote and Wooding, 1966, 1968). I have investigated cellulase activity in differentiating tissues of the sycamore, *Acer pseudo-*

platanus, and in this paper present evidence that cellulase is involved in both xylem and phloem differentiation.

Although there has been little work on higher plant cellulase, fungal cellulolytic enzymes have been studied in some detail. A synergistic complex of enzymes has been found in fungal culture filtrates falling into two main classes; C_1 enzymes attack native cellulose rendering the molecules susceptible to hydrolysis by C_x enzymes, which are unable to attack unmodified cellulose; they can conveniently be assayed using soluble cellulose derivatives as substrates (Gascoigne and Gascoigne, 1960; Wood, 1968). It is conceivable that higher plants could have an analogous cellulase complex, and I have measured and compared the cellulase activities of xylem, phloem and abscission zone tissue of *Acer pseudoplatanus* with this possibility in mind.

Materials and Methods

Young trees of *Acer pseudoplatanus* L. growing in Madingley Wood, near Cambridge, were used as the source of plant material. Cambial, xylem and phloem tissues were obtained in June and July 1969 by cutting down trees, sawing them into logs and then in the laboratory stripping off the bark and separating the cambium and xylem tissues as described by Rubery and Northcote (1968). Phloem samples were obtained by scraping the inside of the bark with a spatula. The samples were weighed, and then homogenized in about ten times their volume of ice-cold 0.01 M phosphate buffer, pH 6.5, containing 0.4 M mannitol. The homogenates were passed through nylon mesh, and were centrifuged at 2,000 g to remove cell debris. In some experiments centrifugation at 25,000 and 100,0000 g was carried out. Samples were tested for cellulase activity immediately, and in some cases stored at —20° C; it was found that enzyme activity declined only slightly under these conditions. Triton X-100 (Rohm & Haas Co., Philadelphia) was added to some homogenates to give a final concentration of 0.1% (v/v).

Leaves were collected at intervals in September and October 1969, a period covering their senescence. It was found that when leaves were broken off, even before senescence began, they broke across the abscission zone, half of which was consequently at the base of the petiole of the detached leaf. At the time of the leaf fall, two classes of leaf were collected, those which fell off when the branch was gently shaken, described here as "falling leaves", and those senescent leaves which remained attached after the branch had been shaken ("attached leaves"). Abscission zone samples were obtained by cutting of the basal 2 mm of the petioles with a razor blade. Slices (2 mm) were also taken from the middle of the petioles for comparison. The samples were weighed and homogenized as above.

Sycamore callus tissue was isolated and has been maintained in this laboratory as described by Stoddart *et al.* (1967) and was kindly supplied by Dr. D. H. Northcote. It was homogenized in the same way as the other tissues.

Bleeding sap was collected from trees of *Acer pseudoplatanus* and *Betula populifolia* Marsh in March 1970, and guttation fluid was collected from seedlings of *Avena sativa* L., *Triticum vulgare* Vill. and *Hordeum vulgare* L. as described by Sheldrake and Northcote (1968). The xylem exudate from decapitated plants of *Lycopersicon esculentum* Mill. growing in pots was collected from rubber tubing attached to the stumps of the plants. These samples were assayed immediately after collection.

Cellulase activity was assayed viscometrically using carboxymethyl cellulose (CMC) as substrate. The CMC, Na salt, was obtained from British Drug Houses, Ltd. The reaction mixtures, methods and calculation of units were the same as described by Sheldrake and Moir (1970) except for the fact that incubations were carried out in a glass sided water bath at 35° C, and the units were based on the difference in flow time 5 and 30 minutes after mixing the enzymes and substrate. McIlvaine citric acid/sodium phosphate buffer was used for work on pH optima and the pH of reaction mixtures was checked after incubation, using a Radiometer pH meter. The cellulase activity of the xylem sap samples was too low to be measured under these conditions: instead incubations were carried out in test tubes in the presence of toluene at 37° C for 24 hours. The outflow times between two fixed marks of a standard pipette were measured, and results expressed as the percentage decrease in flow time.

Cellulase activity was also measured by the release of reducing sugars, estimated by the Nelson-Somogyi method (Bell, 1955). Whatman No. 1 cellulose powder and swollen cellulose were used as substrates. The latter was prepared by dissolving cellulose powder in syrupy phosphoric acid at 4° C and then adding this to a large excess of cold water. The swollen cellulose thus obtained was washed thoroughly before use.

Dialysis was carried out in Visking dialysis tubing against 0.01 M phosphate buffer, pH 6.5, at 4° C overnight. Only a small loss of enzyme activity occurred during this procedure.

Dry weights were measured on samples oven-dried at 60° C for 3 days. Soluble protein was estimated by the biuret method (Gormall *et al.*, 1949).

Results

Cellulase Activity in Callus Tissue

Low cellulase activity could be detected in homogenates of sycamore callus tissue. The enzyme was assayed at pH 6.0. The amount found varied between 0.3 and 1.2 units per g fresh weight. Treatment of the homogenates with the detergent Triton X-100 had little or no effect on cellulase activity.

Cellulase Activity in Cambium, Xylem and Phloem Tissue

Cellulase activity in these tissues is shown in Table 1. In whatever way the results are expressed the cellulase activity in xylem and phloem tissue is higher than in cambial tissue and always highest in the phloem samples.

It was found in preliminary experiments that treatment of homogenates with the non-ionic detergent Triton X-100 led to an increase in cellulase activity. The ratios of enzyme activity in homogenates to which Triton X-100 had been added and in untreated controls are shown in Table 2. The ratios of activity at two different pHs, 6.0 and 7.0, were also determined. It can be seen that in xylem and cambial homogenates, treatment with the detergent led to a considerable increase in cellulase activity; a smaller increase also occurred with phloem samples. The

Table 1. *Cellulase activity in cambium, xylem and phloem tissue*
The enzyme activities of Triton-treated homogenates were assayed at pH 6.0.

	Cambium	*Xylem*	*Phloem*
Cellulase units/g fresh weight	4.5	9.0	55.0
Relative cellulase activity:			
Fresh weight basis	1.0	2.0	12.4
Dry weight basis	1.0	1.4	6.7
Protein basis	1.0	2.1	11.4
Cell basis[a]	1.0	3.8	—

[a] Calculated from data in Thornber and Northcote (1961)

Table 2. *Cellulase activity: ratios under different conditions*

	Cambium	*Xylem*	*Phloem*
Activity at pH 6.0: $\frac{\text{homogenate} + \text{Triton}}{\text{homogenate} - \text{Triton}}$	4.85	3.10	1.15
homogenate − Triton: $\frac{\text{activity at pH 6.0}}{\text{activity at pH 7.0}}$	0.98	1.19	0.76
homogenate + Triton: $\frac{\text{activity at pH 7.0}}{\text{activity at pH 6.0}}$	1.78	1.32	0.97
100,000 g pellet: $\frac{\text{activity at pH 6.0}}{\text{activity at pH 7.0}}$	1.80	2.20	1.43

ratios of activity at the two pHs are different in the different tissues, and are also changed in any given tissue by Triton treatment of the homogenate. These results suggest that two or more forms of enzymic activity are present and that the proportions of activities are different in the different tissues, and also in the soluble and Triton-releasable fractions of the same tissue.

Differential centrifugation was used to study the distribution of enzymic activity in different fractions of the homogenates. They were first spun at 25,000 g for 10 minutes and the resulting supernatant was then spun at 100,000 g for 2 hours. The pellets were resuspendend in

Table 3. *Distribution of cellulase activity in fractions separated by centrifugation.*
Enzyme assays were carried out at pH 6.0. The results represent the total units in each fraction of a homogenate of 10gms of tissue.

Fraction	*Cambium*	*Xylem*	*Phloem*
25,000 g pellet	15	21	14
100,000 g pellet	21	64	23
100,000 g supernatant	28	47	445
Percentage of total activity in pellets	57	65	8

buffer. It was found that their cellulase activities were increased by the addition of detergent; they were also increased by freezing and thawing several times, although not to so great an extent. The addition of Triton X-100 to the 100,000 g supernatant had no effect on the enzymic activity. The cellulase activities of the resuspended pellets, treated with detergent, and of the 100,000 g supernatants are shown in Table 3. The majority of sedimentable cellulase activity in all cases is in the 100,000 g pellet.

The variation of cellulase activity with pH was measured in some detail both in the presence and absence of Triton X-100. A typical curve for a phloem homogenate in the absence of Triton is shown in Fig. 1. The main optimum is at pH 7.1, but shoulders on the curve can be seen at around pH 5.3 and 5.9; this experiment was repeated several times and these shoulders were always found. Triton-treated homogenates gave almost identical curves except for the fact that the shoulder at pH 5.9 was considerably accentuated. Xylem homogenates showed a much more pronounced effect of Triton on the shape of the pH curves (Fig. 1). In the absence of detergent a broad, flat-topped curve was obtained. In each of four separate experiments, three small peaks could be discerned, at about pH 5.3, 5.9 and 6.5. After treatment with Triton, a pronounced optimum at pH 5.9 was found, with a distinct shoulder at about pH 5.3. These results, together with those in Tables 2 and 3, suggest that enzymic activity at pH 5.9, and possibly at pH 5.3, is localized in structures sedimenting mainly at 100,000 g and that this activity can be released by treatment with detergent. This was tested by measuring the pH optimum of the cellulase activity contained in the 100,000 g pellets. In Fig. 2 it can be seen that in all cases the pH optimum is about 5.9. The pellets had not been washed, and would therefore be expected to be contaminated with supernatant enzyme activity; this is high in the case of phloem and could explain why the enzyme ectivity in the phloem pellet does not fall off so sharply towards neutrality as in the other samples.

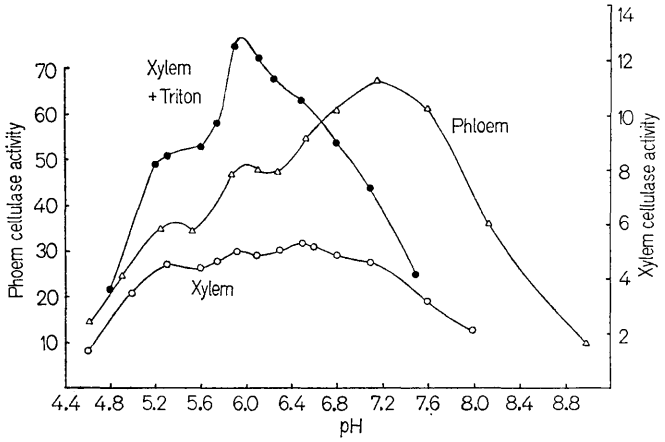

Fig. 1. Effect of pH on cellulase activity of phloem and xylem homogenates. Supernatants of homogenates centrifuged at 100,000 g for 2 hours were used for the enzyme assay. The activities shown correspond to 1 g fresh weight of tissue

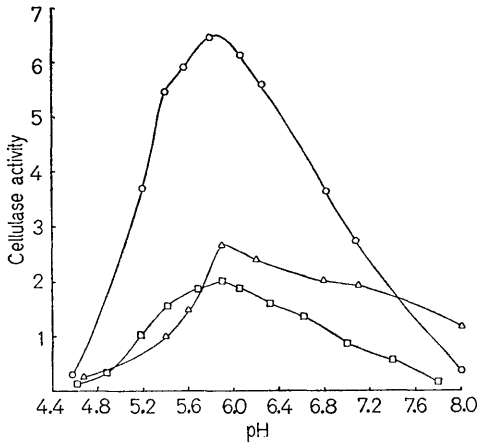

Fig. 2. Cellulase activity in 100,000 g pellets of cambium —▫—, xylem —○— and phloem —▵— homogenates. The activities shown correspond to 1 g fresh weight of tissue

The activities of Triton treated xylem and phloem homogenates were measured using cellulose powder and swollen cellulose as substrates. The homogenates were first dialysed to lower the reducing sugar blanks; dialysis led to only a small decrease in cellulase activity (estimated viscometrically). The results in Table 4 show that the ratios of activity against the two substrates were different: the xylem homogenate was relatively more effective in attacking the cellulose powder.

Table 4. *Cellulase activities with different substrates*

Triton-treated homogenates which had been dialysed for 14 hours were used. The reaction mixture contained 1 ml enzyme, 0.5 ml 0.2 M phosphate buffer 6.0, 0.5 ml of 5 mg/ml suspension of the substrate, and 0.2 ml 0.5 M sodium fluoride. Incubations were carried out at 35° C for 24 hours under toluene. Reaction mixtures were centrifuged and samples of the supernatants used for reducing sugar estimations. Enzyme and substrate blanks were subtracted from the results. All samples were incubated and estimated in duplicate.

	Phloem	*Xylem*
Units/ml: viscometric assay with CMC	5.8	2.2
μg glucose equivs. released/ml enzyme from cellulose powder	5.0	8.0
μg glucose equivs. released/ml enzyme from swollen cellulose	27.0	11.6
Ratio: $\frac{\text{swollen cellulose}}{\text{cellulose powder}}$	$\frac{5.4}{1}$	$\frac{1.5}{1}$

Table 5. *Cellulase activity in xylem sap*

The reaction mixtures consisted of 1 ml xylem sap, 1 ml 0.2 M phosphate buffer pH 6.0 and 1 ml 1% CMC. Incubations were carried out for 24 hours at 35° C. The results are expressed as the percentage decrease in flow time between fixed points of a standard pipette.

Sap sample	Cellulase activity	Sap sample	Cellulase activity
Control	0	Tomato	38
Boiled sap	<1	Barley	12
Sycamore	9	Oat	7
Birch	4	Wheat	17

Cellulase Activity in Xylem Sap

Xylem sap contains a number of hydrolytic enzymes in dilute solution (Sheldrake and Northcote, 1968). Any cellulase present would be expected in low amounts and consequently 24 hour incubations were carried out, instead of the usual 30 minutes. In addition to the bleeding sap of *Acer pseudoplatanus*, the bleeding sap of birch (*Betula populifolia*) and tomato (*Lycopersicon esculentum*) and guttation fluid of oat (*Avena sativa*), wheat (*Triticum vulgare*) and barley (*Hordeum vulgare*) were examined. Table 5 shows that definite cellulase activity was detected in all samples.

The pH optimum of the cellulase of *Acer pseudoplatanus* bleeding sap was in the range 5.8—6.1. The low activity did not permit a more accurate determination than this.

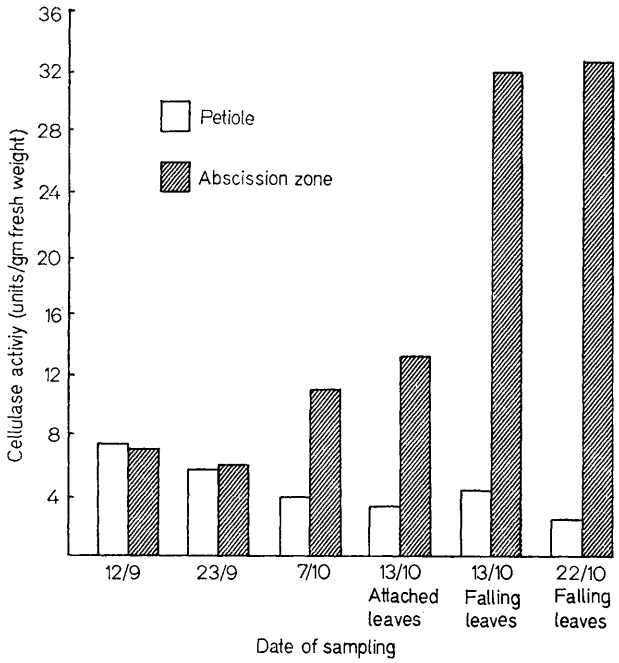

Fig. 3. Cellulase activity in petioles and abscission zone tissue

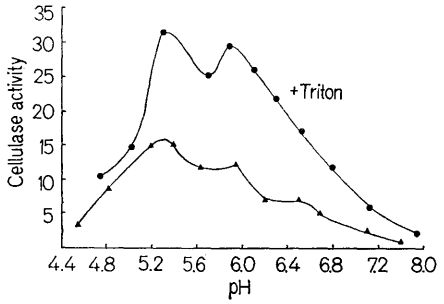

Fig. 4. Effect of pH on activity of abscission zone cellulase. The activities shown correspond to 1 g fresh weight of tissue

Cellulase Activity in the Abscission Zone of Leaves

Measurements of cellulase activity in the abscission zone and in petiolar tissue over a period of several weeks leading up to abscission are shown in Fig. 3. The cellulase activity in petiolar tissue declined steadily, in contrast to the abscission zone where a considerable increase in cellulase activity occurred, reaching the highest levels in leaves which were actually abscinding.

The cellulase activity of abscission zone homogenates was considerably increased by Triton treatment. There were two pH optima in untreated homogenates, at about pH 5.3 and 5.9 (Fig. 4); both of these were increased by Triton treatment. Most of the Triton-releasable cellulase activity could be sedimented by centrifugation at 25,000 g for 10 minutes; the remainder came down at 100,000 g.

Discussion

The higher levels of cellulase in differentiating xylem and phloem tissue than in cambial tissue suggest that cellulase is involved in both xylem and phloem differentiation. The callus tissue, in which no vascular differentiation occurs, contains very low cellulase levels. Although the xylem and phloem tissues are composed of more than one cell type, the most likely explanation of the results is that the cellulase is present in the differentiating xylem and phloem elements themselves, where it is involved in the removal of wall material. The lower level of cellulase in xylem than phloem tissue might be explained by the fact that when a xylem cell has finished differentiating, its lumen becomes a conducting channel for xylem sap, the upward flow of which will sweep away the digested remnants of the cell and the enzymes concerned with cell differentiation. Various metabolites and enzymes probably derived from newly differentiated xylem cells can in fact be detected in xylem sap (Sheldrake and Northcote, 1968). The presence of cellulase in bleeding sap and guttation fluid (Table 5) is consistent with this interpretation. The detailed ultrastructural studies of O'Brien and Thimann (1967) and O'Brien (1970) show that the remains of hydrolysed wall material that can be observed in differentiated xylem cells probably contain some cellulose which has not been more than partially digested during cell differentiation. This could be because cellulose is hydrolysed only slowly and the enzymes involved are carried away from the xylem cells as soon as they become functional, before complete digestion of the cellulose has had time to take place. In the sieve elements of the phloem, on the other hand, the contents of the differentiated cells remain, and thus any cellulase involved in phloem differentiation would be likely to persist, although presumably no new cellulase can be made in mature sieve tubes since they lose their nuclei and ribosomes during differentiation (Northcote and Wooding, 1968). By contrast, in latex vessels, which contain even higher amounts of cellulase than phloem tissue, the nuclei of the differentiated cells persist and cellulase continues to be formed in latex vessel systems long after their differentiation (Sheldrake and Moir, 1970).

The low levels of cellulase found in petiolar and in abscission zone sections of non-senescing leaves could be due to continuing vascular

differentiation within the petioles. As leaf senescence progresses, the amount of cellulase in abscission zone sections increases while petiolar sections show a decline. The greatest increase in abscission zone cellulase must occur shortly before abscission since senescent leaves which are still attached have considerably less cellulase in their abscission zones than do leaves which are actually abscinding (Fig. 3).

The curves in Figs. 1, 2 and 4 and the results in Tables 2 and 4 suggest the existence of several different cellulases, or several different forms of the same cellulase. However if different forms of the same enzyme are involved they must be quite stable since they can exist under different conditions and persist in the presence of each other. The simplest and most probable interpretation of the results seems to be that an enzyme with an optimum at pH 5.9 and possibly a second enzyme most active at pH 5.3 are present in a predominantly membrane-bound form in cambial, phloem, xylem and abscission zone tissue. In phloem tissue a cytoplasmic, non-membrane bound enzyme with a pH optimum of 7.1 is present; this is not found in xylem and abscission zone tissue, but in xylem tissue a cytoplasmic enzyme with an optimum at pH 6.5 might be present instead. It should be possible to test these interpretations by separating the enzyme activities experimentally.

It was suggested by Sheldrake and Moir (1970) that if plants have a synergistic cellulase complex analogous to that of fungi, it might be expected that the enzymes concerned with the initial degradation of cellulose would be exported into the cell walls, where their substrate is to be found. The solubilized and partially degraded cellulose molecules released by these enzymes could then move across the cell membrane and be hydrolysed by intracellular cytoplasmic cellulases. The Triton-releasable cellulase found in xylem, phloem and abscission zone tissue could represent part of an enzyme complex concerned with the initial degradation of cellulose, exported in small membrane-bound vesicles to an appropiate region of the wall. In sieve tubes further degradation of the solubilized cellulose could then occur in the cytoplasm: the contents of sieve tubes are slightly alkaline (cf. Tammes and van Die, 1964), and the cytoplasmic cellulase has an optimum of pH 7.1. Perhaps a similar process occurs in xylem differentiation, where a cytoplasmic enzyme with a slightly more acid optimum (pH 6.5) could be involved; but the death and disorganisation of xylem cells during differentiation means that any hydrolytic enzymes present will be able to attack any chemically susceptible walls no longer physically protected by the cell membrane. Not only are end walls hydrolysed, but also primary wall material between bands of lignified secondary thickening (O'Brien and Thimann, 1967; O'Brien, 1970). In the cells of the abscission zone, a weakening of cell walls sufficient to cause abscission occurs, and the export of cellulases

into the wall is almost certainly involved in bringing this about. But a complete digestion of wall material would be of no advantage, and no cytoplasmic cellulase comparable to that of sieve tubes or latex vessels is found.

It has been proposed by Fan and Maclachlan (1966) that cellulase is responsible for IAA-induced expansion growth of cells by weakening cell walls. It was found that treatment of decapitated pea epicotyls with high concentrations of indole acetic acid (IAA) in lanoline paste led both to a swelling of the tissue and a considerable increase in cellulase activity over a 3—4 day period. Subsequent papers from the same laboratory have elaborated these results in more detail (e.g. Fan and Maclachlan, 1967; Datko and Maclachlan, 1968; Maclachlan *et al.*, 1968). However, although cell expansion and cellulase activity both occur in response to IAA treatment, there is no evidence that the rise in cellulase activity is the cause of cell expansion. Within the period studied, the IAA treatment not only leads to cell expansion but also to xylem differentiation and lateral root initiation (Scott, 1938). The rise in cellulase activity which follows the application of IAA could therefore be due to the induction of vascular differentiation by the treatment. Pea stems can also be caused to swell by ethylene, but in this case the cell expansion is not associated with increased cellulase activity (Ridge and Osborne, 1969). A direct test of the hypothesis that cellulase is involved in cell growth has recently been reported by Ruesinck (1969) who found that treatment of *Avena* coleoptiles with cellulase, both in the presence and absence of IAA, did not lead to increased growth. Thus cellulase may have nothing to do with auxin-induced cell growth, and may only be involved in removing or softening the walls of specialized cells, as in abscission zones, and in xylem, phloem and latex vessel differentiation. The presence of cellulase in homogenates from a wide variety of higher plants (Tracey, 1950; Gascoigne and Gascoigne, 1960; Clark and Stone, 1962) could be explained by its rôle in vascular differentiation in the tissues analysed.

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