Activity of Peptidase in Tobacco-Leaf Tissue in Relation to Senescence

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1. Peptidase extracted with buffer at pH 5.2 from small samples of tobacco-leaf tissue was assayed by measuring the α -amino nitrogen liberated from gelatin at 40° in 1 hr. The rate of hydrolysis was constant during the digestion. 2. The inclusion of sodium thioglycollate (60mm) in the extracting medium increased the activity of the peptidase. 3. As the major products of hydrolysis were peptides, the enzyme is an endopeptidase. 4. The decrease in protein observed in leaves with increasing physiological age and with time after harvest is correlated with increase in specific activity of peptidase in extracts from the leaves.

In the mature tobacco plant the concentration of protein and soluble nitrogen is lower in the older than the younger leaves (Mothes, 1931; Noguchi & Tamaki, 1962); after harvest, protein nitrogen decreases with time but soluble nitrogen increases (Vickery, Pucher, Wakeman & Leavenworth, 1937; Weinstein & Porter, 1962). Changes in activity of peptidase are examined here in relation to the decrease in concentration of protein with increase in physiological age and with time after harvest. This work precedes a study of the mechanism of action of 6-furfurylaminopurine (kinetin) in delaying senescence in leaf tissue of tobacco.

Previous assays of peptidase extracted from leaf tissue required digestion for at least 24 hr. or preparation of acetone-dried powders. When thioglycollate was added to the extracting medium, as in the method reported here, accurate assays required 1 hr. only. The properties of peptidase reported here confirm and extend the work of Tracey (1948).

MATERIALS AND METHODS

Tobacco plants (*Nicotiana tabacum* L., cultivar Virginian Gold) were grown in a glass-house at not less than 13°. The supply of N to the plants was lowered by growing them in small pots, which produces plants high in peptidase per unit leaf protein (Holden & Tracey, 1948) and low in soluble N. Leaves were harvested at 2–3 months.

Disks 1.3 cm. in diameter were cut from leaves with a cork-borer, the main veins being avoided. Replicate samples contained equal numbers of disks (16–26) from each leaf; the approximate mass of each disk was 30 mg.

Bacto gelatin was supplied by Difco Laboratories Inc., Detroit, Mich., U.S.A.; casein (Hammarsten) and haemoglobin were supplied by British Drug Houses Ltd. and crystalline serum albumin by Commonwealth Serum Laboratories, Parkville, Victoria, Australia.

Solutions of sodium thioglycollate were prepared by titrating thioglycollic acid (L.R. grade; British Drug Houses Ltd.) to the pH required with 2N-NaOH and adjusting to 1.2M. Buffer solutions were prepared from appropriate mixtures of Na₂HPO₄ (0.2M) and citric acid (0.1M).

When required, the least significant difference between two mean values was calculated by a *t* test after analysis of variance.

Extraction of peptidase. Unless stated otherwise, the extracting medium contained sodium thioglycollate (60mm) in buffer at pH 5.2. Samples were extracted by grinding for 3 min. with a glass pestle and mortar, approximately 3 ml. of extracting medium being used per g. of leaf tissue. Addition of acid-washed sand to the extracting medium during grinding did not increase the amount of peptidase extracted. The enzyme solution was recovered by centrifuging at 15 000g for 45 min., and was dialysed against 15 vol. of extracting medium for 22 hr. with one change of medium in this time. The precipitate remaining after centrifugation was retained for estimation of chlorophyll. Dialysis removed 95% of the α -amino N originally present in the enzyme solution. All operations described above were carried out in a cold room at 1°.

Estimation of activity of peptidase. Unless stated otherwise, 0.5 ml. of enzyme solution was mixed with 0.5 ml. of 4% (w/v) gelatin dissolved in buffer at pH 5.2, and incubated at 40°. Peptidase was assayed by measuring the rate of increase in α -amino N in the digestion, 0.1 ml. samples being removed for analysis at 2 and 62 min. The extinction after treating the samples with the ninhydrin reagent of Moore & Stein (1948) was measured at 570 m μ in a final volume of 6–10 ml. and the activity of the enzyme expressed as ΔE_{570} . The increase in E_{570} due to thioglycollate was independent of the amount of α -amino N present. Values of ΔE_{570} for each digestion are means of duplicate analyses.

E_{570} of a series of standards containing 5–40 μ g. of acid-hydrolysed gelatin/0.10 ml. was used to convert ΔE_{570} into m μ equiv. of peptide bonds hydrolysed/min. (peptidase units). E_{570} of 100 m μ equiv. of peptide bonds in the gelatin standards was 0.345 unit.

Estimation of α -amino nitrogen in the enzyme solution. α -Amino N was estimated as E_{570} in 0.10 ml. samples of the enzyme solution by the modification of the method of Moore & Stein (1948) used to estimate the activity of peptidase. E_{570} was measured in a final volume of 6.10 ml.

Estimation of protein in the enzyme solution. Ethanol (1.5 ml.; 67%, v/v) was added to 1.0 ml. of enzyme solution in a glass centrifuge tube and the mixture held at boiling point for 2 min. After cooling, the precipitate was recovered by centrifugation and washed twice with 5 ml. of acetone. The final precipitate was dried *in vacuo* and digested in 5.0 ml. of 4N-NaOH at 50° for 2 hr. After cooling, the supernatant solutions were recovered by centrifugation and protein was determined in 1.0 ml. samples by the method of Ellman (1962) with standard solutions of serum albumin.

Estimation of chlorophyll. Chlorophyll in the precipitate remaining after extracting peptidase was extracted with boiling ethanol (80%, v/v) and the concentration per sample of leaf disks expressed as the extinction at 665 m μ (E_{665}).

Chromatography of the products of peptidase digestion. Descending chromatograms were run on Whatman no. 3 filter paper with butan-1-ol-acetic acid-water (4:1:1, by vol.) as solvent. Replicate chromatograms were run for 13, 31 and 47 hr. to detect amino acids with high, medium and low R_F values respectively. Amino acids were detected by spraying the dried chromatograms with ninhydrin (0.5%, w/v, in butan-1-ol) and heating; duplicate chromatograms were treated by the method of Rydon & Smith (1952) to detect peptides.

RESULTS

Effect of including sodium thioglycollate in the extracting medium upon the activity of peptidase. The activity of peptidase prepared without thioglycollate in the extracting medium was low, and the preparations became dark during extraction and incubation. Addition of potassium cyanide (3–60 mm) to the extracting medium did not

prevent darkening or increase activity. The activity was higher when sodium thioglycollate (30 mm) was included in the incubating medium, as observed by Tracey (1948) and Singh (1962), but also when sodium thioglycollate (60 mm) was included in the extracting medium the activity increased by a further factor of 2–4, and darkening was inhibited during extraction and assay (Table 1). Addition of thioglycollate to the digest immediately before adding it to the ninhydrin reagent did not increase the value of ΔE_{570} .

The activity of peptidase was measured in extracts from replicate samples of leaf disks by using extracting media containing sodium thioglycollate (between 1 and 110 mm). The activity with sodium thioglycollate above 10 mm was approximately sixfold that with 0–5 mm-thioglycollate (Fig. 1). Thus the concentration used for assay (60 mm) is well within the optimum range found in this experiment.

Relative activity of peptidase upon different substrates. As some of the substrates tested were relatively insoluble at pH 5.2 extraction and assays were made at pH 7.0. Substrates included fraction I protein prepared from tobacco leaf by the method of Wildman & Bonner (1947). This preparation contained little activity of peptidase. Casein, with the highest ΔE_{570} at pH 7 (Table 2), was relatively insoluble at the optimum pH (pH 5.2) of the enzyme and gave high blank values with ninhydrin. Accordingly, gelatin was used as substrate for subsequent assays.

Table 1. *Effect of sodium thioglycollate in the extracting and incubating medium upon the activity of peptidase from tobacco leaf*

The incubating medium contained 0.5 ml. of enzyme solution, 0.5 ml. of 4% (w/v) gelatin and 0.1 ml. of sodium thioglycollate or water. E_{570} was determined in 0.1 ml. of incubating medium. Activities shown are mean values from duplicate extractions.

Concn. of sodium thioglycollate (mm)		ΔE_{570}	Sp. activity of enzyme	
Extracting medium	Incubating medium		Enzyme protein extracted (mg./ml. of extract)	peptidase (units/mg. of protein)
0	0	0.165	2.25	7.8
0	30	0.402	2.25	18.9
60	30	0.837	2.36	37.8

Fig. 1. *Effect of concentration of sodium thioglycollate in the extracting medium upon activity of peptidase.*

Time-course of the enzymic digestion. The hydrolysis of gelatin during an incubation of 2 hr. is shown in Fig. 2. As the rate of hydrolysis was constant for 1 hr. this time was used for assays rather than the 24 hr. incubations used by previous workers (Tracey, 1948; Brady, 1961; Singh, 1962; Tazawa & Hirokawa, 1963). The rate of hydrolysis calculated after incubation for 24 hr. (over chloroform) with a comparable enzyme preparation was much lower (Table 3).

Effect of pH of extraction and assay upon activity of peptidase. Peptidase was extracted with medium ranging from pH 4 to 7. All extracts were dialysed against extracting medium at pH 5.5. The optimum for absolute activity was at pH 5.2 and above (Fig. 3) but the optimum for specific activity was at pH 4.9, showing that the enzyme protein was extracted preferentially at values below pH 5.2.

Table 2. Activity of peptidase from tobacco leaf tested with various proteins as substrate

All substrates were dissolved in buffer at pH 7.0 to give a concentration of 2% (w/v) in the digestions and were assayed with enzyme extracted at pH 7.0. Substrate was replaced by 0.5 ml. of buffer when the activity upon the endogenous protein was tested. (Activities shown are mean values of duplicate digestions.)

Substrate	Activity of peptidase (ΔE_{570})
Endogenous	0.19
Gelatin	0.87
Serum albumin	0.23
Haemoglobin	0.64
Casein	1.21
Fraction I protein (tobacco)	0.56

Fig. 2. Time-course of hydrolysis of gelatin by peptidase.

Samples of a large batch of enzyme extracted at pH 5.5 were dialysed against extracting medium at a range of values between pH 4 and 7. Activity of peptidase was measured by incubating at the same pH (Fig. 4). As pH 5.2 was optimum for both extraction and assay, subsequent extractions and assays were made at this pH value.

Effect of concentration of enzyme and dialysis upon activity of peptidase. One-half of an enzyme extract was dialysed against extracting medium at pH 5.2 and the other half was held at 1° without dialysis. Both preparations were assayed for activity over a range of dilutions. Dialysis did not

Fig. 3. Effect of pH of extraction upon absolute (○) and specific activity (●) of peptidase. Extracts prepared with media buffered between pH 4 and 7 were dialysed against medium buffered at pH 5.5 for 24 hr. All media contained sodium thioglycollate (60 mm). Assays were made at pH 5.5.

Fig. 4. Effect of pH of assay upon activity of peptidase. Samples of an extract prepared with medium buffered at pH 5.5 were dialysed against media buffered between pH 4 and 7 for 24 hr. All media contained sodium thioglycollate (60 mm). Assays were made at the pH at which the extracts were dialysed. Activities are means of duplicate digestions.

alter the activity or the concentration of protein in the enzyme solution and the rate of the reaction was a linear function of enzyme concentration over the range examined. Dialysis lowered the concentration of α -amino nitrogen in the enzyme solutions and was used in subsequent preparations.

Determination of the Michaelis constant (K_m). The approximate K_m for gelatin (0.9%, w/v) was estimated by the method of Lineweaver & Burk (1934), correcting concentrations of gelatin (s) by adding the concentration of protein in the enzyme solution (0.057%, w/v). The values of s/v calculated with low values of s were neglected, thus reducing error introduced by difference in value of K_m for gelatin and endogenous substrate (Fig. 5).

Products of the hydrolysis of gelatin by peptidase. A concentrated enzyme solution (lg. fresh wt. of tissue/ml. of extracting medium) was mixed with an equal volume of gelatin (4%, w/v) and incubated at 40° for 65 min. Control solutions with gelatin replaced by buffer, or enzyme solution replaced by extracting medium, were incubated in the same manner. ΔE_{570} was measured in all mixtures. After incubation, residual gelatin and enzyme protein was precipitated with ethanol (80%, v/v). The precipitate was removed by centrifugation and α -amino nitrogen determined in duplicate on 0.1 ml. samples of each supernatant solution. The remaining solutions were evaporated to dryness in a rotary film-evaporator and redissolved in 0.625 ml. of 80% (v/v) ethanol. Samples (10 μ l.) of each solution were applied to paper chromatograms and mixtures of the appropriate amino acids were added to each as markers. On the chromatograms

treated with ninhydrin, the intensity of the spots at the positions of the free amino acids did not increase after hydrolysis, but ninhydrin-positive streaks with R_f lower than the slow-running amino acids appeared more intense. New, fainter spots appeared also, with R_f slightly higher than the slow-running amino acids. When replicate chromatograms were treated with the spray for peptides (Rydon & Smith, 1952), the streaks previously detected with ninhydrin appeared intensely black, relatively much darker than they appeared on chromatograms treated with ninhydrin. When E_{570} was measured in the enzyme digestion from which gelatin had been removed by precipitation with ethanol, the value (corrected by appropriate blank values for gelatin and enzyme) had decreased by 60%. This appeared to be from the loss of peptides of high molecular weight which were insoluble in ethanol. As the products of digestion by the peptidase in the first hour appear to be peptides rather than amino acids, the enzyme is an endopeptidase.

Changes in cell size and concentration of chlorophyll per cell with increase in physiological age of leaf. Two samples of five disks were cut from each of a series of leaves from a single tobacco plant. Chlorophyll was estimated in one sample and, in the other, cell volume was calculated by the method of Brown & Rickless (1949). Changes in cell volume, cell number per disk and chlorophyll content per cell

Fig. 5. Determination of K_m for gelatin by the method of Lineweaver & Burk (1934). The concn. of gelatin (s) was corrected by adding the concn. of protein in the enzyme extract (0.057%, w/v). K_m was 0.9% (w/v) gelatin. v , Units/mg. of enzyme protein.

Fig. 6. Changes in cell volume (●), cell number per disk (○) and chlorophyll content per cell (▲) with increase in physiological age of leaf. E_{665} was measured in 25 ml. of 80% (v/v) ethanol. The vertical line shows the least significant difference ($P = 0.05$) between two mean values for cell volume.

Fig. 7. Changes in absolute (○) and specific activity (●) of peptidase and in concn. of chlorophyll (▲) and soluble protein (△) with increase in physiological age of leaf. Disks (20) were ground in 2.1 ml. of extracting medium. E_{665} was measured in 10 ml. of 80% (v/v) ethanol. Vertical lines show the least significant differences ($P < 0.05$) between two mean values for absolute and specific activity of peptidase.

Fig. 8. Changes in concn. of α-amino N (●) and chlorophyll (▲) with increase in physiological age of leaf. Disks (16) were ground in 3.0 ml. of extracting medium. E_{665} was measured in 25 ml. of 80% (v/v) ethanol.

are shown in Fig. 6. Cell volume was smaller in younger leaves and the number of cells in disks cut from them was approximately 25% higher than in the older leaves.

Changes in concentration of chlorophyll, α-amino nitrogen and soluble protein and in activity of peptidase with increase in physiological age of leaf. In two experiments, samples of disks were prepared from a series of leaves from a single plant. Chlorophyll content was measured in both and serves to relate the two experiments. In the first experiment, absolute and specific activity of peptidase was measured (Fig. 7), and, in the second, concentration of α-amino nitrogen (Fig. 8). Concentration of chlorophyll, α-amino nitrogen and soluble protein decreased with increasing physiological age; absolute activity of peptidase remained constant, but the specific activity increased.

Changes in concentration of chlorophyll, α-amino nitrogen and soluble protein and in activity of peptidase in leaf disks after harvesting. Samples of disks were floated with the abaxial surface upwards on 10 ml. of glass-distilled water in Petri dishes at 25° in darkness; the water was replaced daily. At

Fig. 9. Changes in absolute (○) and specific activity (●) of peptidase and in concn. of α-amino N (■), chlorophyll (▲) and soluble protein (△) of leaf disks with time after harvest. Disks (25) were ground in 3.0 ml. of extracting medium. E_{665} was measured in 25 ml. of 80% (v/v) ethanol. All results are means of duplicate extractions. Vertical lines show the least significant differences ($P < 0.05$) between two mean values for absolute and specific activity of peptidase.

intervals of 2 days, samples of disks were blotted lightly, weighed and analysed (Fig. 9). The concentration of α -amino nitrogen increased and that of chlorophyll decreased throughout the experiment. The concentration of soluble protein decreased, and specific activity of the peptidase increased by a greater factor than absolute activity. All changes were significant after 2 days.

DISCUSSION

The specific activity of the preparation described here is substantially higher than previous preparations of unpurified peptidase from leaves (Table 3). The short time of assay used (1 hr.) does not account entirely for the higher rate, as this is still 15-fold the rate observed by Tracey (1948) when both are compared with an assay lasting 24 hr. Inclusion of thioglycollate in the extracting medium is the most important factor for extracting peptidase with high activity. Cysteine, metabisulphite and dithionite added to the extracting medium increased the activity also. Tracey (1948) and Singh (1962) observed that peptidase was more active when thiols were included in the incubating medium, though Brady (1961) found thiols effective with endogenous substrate only. Preparation of an acetone-dried powder appeared an alternative method of preparing enzyme with high activity from leaves of the tea plant (Sanderson & Roberts, 1964).

Activity of the enzyme expressed per unit fresh weight did not change significantly with physiological age (Fig. 7), and if expressed per cell would tend to increase rather than decrease with age of leaf, as cell volumes were higher in older leaves (Fig. 6). As the soluble protein decreased, specific

activity of peptidase increased steadily with physiological age of the leaf (Fig. 7) while chlorophyll and α -amino nitrogen decreased (Fig. 8). After harvest, peptidase per g. fresh weight increased for 4 days, while soluble protein decreased, accounting for the marked rise in specific activity (Fig. 9). The decrease in chlorophyll and increase in α -amino nitrogen reflect the degradation of protein after harvest.

As specific activity of the peptidase increased with physiological age and with time from harvest, peptidase protein appears more stable than the bulk of soluble protein. Similarly, the activity of phosphatase, β -fructofuranosidase (invertase) and peroxidase remained constant in tobacco leaf for 7 days after harvest (Axelrod & Jagendorf, 1951). As previous workers have shown that total protein of tobacco leaf decreased with increase in physiological age (Mothes, 1931; Noguchi & Tamaki, 1962), and with time from harvest (Vickery *et al.* 1937), the effect is unlikely to be due to a change in the proportion of the leaf protein soluble in buffer at pH 5.2. Stability of peptidase is consistent with nitrogen moving from old to young leaves (Watson & Petrie, 1940) and with the pattern of senescence in leaves before and after harvest.

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Table 3. Comparison of activity of peptidase extracted from leaf tissue by other workers with the activity reported in this paper

Activities found by other workers in unpurified preparations with gelatin as substrate are converted to 40° and to concn. of gelatin of 2% (w/v), assuming where necessary that $Q_{10} = 2$ and that $K_m = 0.9\%$ (w/v). The activity of the peptidase quoted from the work described in this paper is modal.

Reference	Species	Time of incubation (hr.)	Sp. activity of peptidase (units/mg. of enzyme protein)
Tracey (1948)	<i>N. tabacum</i> L.	24	1.4
Brady (1961)	<i>Trifolium repens</i> L.	24	0.4
This paper	<i>N. tabacum</i> L.	24 1	21.2 57.0