The Leaf Protease of *Trifolium repens*

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With the possible exception of bromelin (Murachi & Neurath, 1959), the proteolytic enzymes of the green leaves of non-latex-bearing plants have been studied very little in a direct and quantitative manner. In fact, a paper by Tracey (1948) appears to be the only report of detailed experimentation on this subject. This author studied the properties of a partially purified protease from the leaves of the tobacco plant, and determined protease activity, under standard conditions in the leaf sap of a number of other plants. Tracey concluded that the leaf proteases formed a group separated from the enzymes of the latex-bearing plants and from bromelin by their very much lower activity, but similar in being activated by reducing agents and having, for protein substrates, a pH optimum in the range 5–7.

In recent years, there has been considerable interest in the changes in nitrogen distribution in starving leaves. A rapid breakdown of protein to non-protein forms of nitrogen occurs during the processes of wilting and ensilage (e.g. Kemble & Macpherson, 1954*b*; Kemble, 1956; Brady, 1960). Macpherson (1952*a*), from a comparison of the rate of breakdown of protein and the rate of fall of pH in grass sap, concluded that attainment of pH 4·3 in ensilage prevents further proteolysis. Comparatively little is known, however, of the factors directly affecting proteolysis in plant material. Obviously, more direct investigation of the properties of the enzyme systems involved is desirable.

This paper reports observations made on the proteolytic system in plants of *Trifolium repens* (white clover). The data refer to the clover enzyme acting on endogenous substrate and to its action on gelatin added as substrate.

MATERIALS AND METHODS

Preparation of crude enzyme system. White clover (*Trifolium repens* L.), Irrigation White strain, was grown in field plots at Highett, Victoria. Samples of clover were harvested about an inch above ground level from various places in the field plot. These samples were bulked, checked for botanical purity and freed from dead or diseased material.

Juice was extracted by compressing the mixed leaflets and petioles with a spiral auger within the cone-shaped body of a mechanized juice-extraction unit (Fowlers Vacola Manufacturing Co. Ltd., Hawthorn, Victoria). The juice was filtered through a perforated stainless-steel plate and then through cloth. The fibre was thus removed from the disintegrated cells; most of the chloroplasts were recovered apparently undamaged. After keeping the mixture overnight at −20°, coarse debris and soil particles were removed from the juice by centrifuging for 2 min. at 2400*g*. After it was found that further centrifuging increased the specific activity of the juice (see Table 1), the period was increased to 10 min. When preliminary freezing was omitted much less material was removed in the centrifuge, and aggregation of particles which occurred during incubation made subsequent sampling difficult.

Protease activity was also determined on individual plants grown in small pots in the glasshouse. Juice was extracted from these plants with a small plant press (Pirie, 1956), and the crude enzyme prepared as described above.

Concentration of enzyme. The method of enzyme concentration used by Tracey (1948) was modified by the inclusion of a dialysis step. Two modifications of the concentration procedure are compared in Table 2. Concentration resulted in a fivefold increase in the specific activity of the protein, and in the removal of small molecules which were possible sources of interference in the analytical procedures.

A large batch of juice was treated according to schedule B (Table 2), and the enzyme solution concentrated in a forced draught at 2°. The concentrated enzyme was divided into small lots and stored at −20°. This preparation showed no loss of activity over a period of 9 months.

Incubation media. Crude preparations were examined for action on endogenous substrate by adjusting to pH 6·0 (original pH 5·6–6·1), and incubating 5 ml. of juice with 1 ml. of a solution of activator or of inhibitor or 1 ml. of water. Activity on gelatin substrate was determined in a

Table 1. *Proteolytic activity in centrifuged juice samples*

Proteolytic activity is expressed as increase in α-amino nitrogen (mg./mg. of clover nitrogen/ml.) during incubation for 5 hr. at 45° in a system containing 18 mg. of gelatin, 0·0375M-disodium hydrogen phosphate, 0·0125M-potassium dihydrogen phosphate, 0·02M-sodium thioglycollate and a clover-nitrogen range from 2·72 to 1·74 mg./ml. The pH was 6·0.

Centrifuging treatment	Proteolytic activity
Nil	0·010
2 min. at 500*g*	0·012
5 min. at 1000*g*	0·013
10 min. at 2400*g*	0·017

Table 2. *Partial purification of clover-juice protease*

Data refer only to the supernatant fractions. Specific activity was in all cases less than 100 in the precipitates. In each schedule, the recovery of protease units in the purified fraction was in the region of 20%. (For definition of protease units see text.)

Treatment	Protease (unit/ml.)	Protein N (mg./ml.)	Protease (units/g. of protein N)
Schedule A			
(1) Crude enzyme	0·400	4·00	100
(2) Heated, 10 min. at 55°	0·265	1·07	248
(3) 5% (v/v) Ethanol, pH 4·2	0·207	0·75	274
(4) Dialysed, 16 hr. at 4°, against 0·5 mM-ethylene-diaminetetra-acetate	0·084	0·19	443
(5) Dialysed, 16 hr. at 4°, against water	0·093	0·19	487
Schedule B			
(1) Crude enzyme	0·400	4·00	100
(2) Dialysed, 48 hr. at 4°, against water (two changes)	0·146	0·56	259
(3) Heated, 10 min. at 55°	0·147	0·43	343
(4) 5% (v/v) Ethanol, pH 4·2, then dialysis against water for 4 hr. at room temp.	0·082	0·15	537

medium with 1 ml. of enzyme in a final volume of 5 ml., with a gelatin concentration of 24 mg./ml., disodium monohydrogen phosphate 0·0375 m-mole/ml. and potassium dihydrogen phosphate 0·0125 m-mole/ml.; pH 6·0. The pH was adjusted at the incubation temperature (normally 45°). The gelatin used was obtained from Townson and Mercer, Melbourne, Victoria, and material from the same batch was used in all experiments.

After the addition of sufficient chloroform or toluene to saturate the medium, samples were taken for determining the zero-time level of α-amino nitrogen. Assays were carried out in an atmosphere of nitrogen in Thunberg tubes incubated in a water bath. After incubation the media were mixed and sampled for analysis. When observations were made at a number of time intervals, larger lots of media were incubated in conical flasks and sampled as required.

Protease activity was expressed as the increase in the α-amino-nitrogen concentration. Unit activity is defined as an increase in α-amino nitrogen of 1 mg. during incubation for 24 hr. at 45°, at pH 6·0. When enzyme samples, previously immersed for 2 min. in a boiling-water bath, were used in the assay conditions no activity could be demonstrated.

Although a gas phase of nitrogen was always used in assays, there was little evidence that this was really necessary. Assay results were the same in nitrogen or hydrogen, and differed in an atmosphere of oxygen only when the juice contained no added substrate and no thioglycollate.

Analytical methods

Total nitrogen. Total nitrogen was determined by a semi-micro-Kjeldahl procedure (McKenzie & Wallace, 1954).

Soluble nitrogen. For the determination of soluble nitrogen 10 g. wet wt. of minced tissue or of crude enzyme was extracted for 1 hr. under reflux with ethanol of final concentration 80% (v/v). The extract was removed by filtration and the residue twice more extracted under reflux with 80% (v/v) ethanol. Extracts and washings of the residue were made to 500 ml., and the nitrogen content was estimated by the micro-Kjeldahl procedure.

Soluble non-protein nitrogen. Soluble non-protein nitrogen was that portion of the soluble nitrogen soluble in cold 10% (w/v) trichloroacetic acid.

Steam-volatile nitrogen. Steam-volatile bases in the soluble-nitrogen extracts were determined by steam-distillation of 10 ml of extracts for 10 min. (25 ml. of distillate) in the presence of borate buffer, pH 9·5.

Ammonia nitrogen. Ammonia was determined by a microdiffusion technique (Conway, 1957).

Amide nitrogen. Amide nitrogen was expressed as the increase in ammonia nitrogen after heating for 3 hr. at 100° in N-H_2SO_4.

α-Amino nitrogen. For the determination of α-amino nitrogen the copper-chelation method of Pope & Stevens (1939) as modified by Schroeder, Kay & Mills (1950) was used, with the exception that the titration method of Sully (1955) was employed. The α-amino-nitrogen content was calculated assuming 2 atoms of α-amino nitrogen/atom of copper titrated.

The Pope & Stevens (1939) reagent shows considerable reactivity with peptides and some proteins, including gelatin. The supernatant of a sample to which ethanol had been added to a final concentration of 80% (v/v) gave an appreciably lower α-amino-nitrogen titre than did the sample before treatment. However, when comparisons were made of the increment during incubation, as indicated by α-amino-nitrogen determinations on untreated and ethanol-treated samples, the two values always corresponded very closely. Analysis was usually made without ethanol treatment. Enzyme action was stopped by placing the diluted sample in a boiling-water bath for 2 min. This treatment was without effect on the apparent α-amino-nitrogen content. After boiling, all samples were maintained with a slight excess of iodine for about 30 min., and then excess of iodine was removed with 0·004N-thiosulphate. The iodine treatment served to minimize interference during the subsequent iodimetric determination of the solubilized copper. During extensive use of the method under these conditions the coefficient of variation due to analysis has always been well below 1%.

A number of substances tested as inhibitors were found to interfere with the copper-chelation method. In some cases the preliminary iodine treatment removed this inter-

ference. In other cases the chloramine-T method (Kemble & Macpherson, 1954*a*) was used to estimate α-amino nitrogen. This method gave substantially lower values for α-amino-nitrogen concentration than the Pope & Stevens method, and the increment due to incubation was usually about 80% of that indicated by the copper method.

Tracey (1948) found that the only substance present in tobacco-leaf extracts which was likely to invalidate the use of the Pope & Stevens (1939) method as an index of proteolysis was citric acid. In the present work citric acid has not been found in the clover samples either before or after incubation in amounts sufficient to cause significant interference.

Peptide nitrogen. For determination of the peptide-nitrogen content, samples of the media were deproteinized by ethanol (80%, v/v), evaporated almost to dryness in a boiling-water bath and then heated under reflux in 6N-HCl at 110° for 24 hr. The acid was removed under vacuum at 45° and the hydrolysate was dissolved in borate buffer, pH 9·5. α-Amino nitrogen was determined on the deproteinized samples by the Pope & Stevens (1939) procedure, before and after hydrolysis, and the peptide nitrogen was obtained by difference.

Since peptides in general chelate one atom of copper per molecule as against the one half-atom bound per molecule of free amino acid (e.g. Spies & Chambers, 1951), an error is involved in estimates of mixtures containing free and bound α-amino nitrogen. The most certain estimate is of extracts after hydrolysis, when the conversion factor is completely appropriate. Before hydrolysis, however, the number of molecules binding copper will be overestimated by an amount dependent on the proportion of peptide molecules. As a result the α-amino nitrogen released by hydrolysis will be an underestimate of peptide nitrogen, the extent of the error being dependent on the chain length of the peptides involved.

RESULTS

In Table 3, a comparison is made of the changes in nitrogen distribution in clover leaves ensiled over chloroform, and in a comparable juice sample incubated over chloroform. The changes in distribution are qualitatively the same, and also show remarkable quantitative agreement. It follows that juice samples reflect the pattern of nitrogen distribution found in clover leaves autolysing anaerobically, and therefore differ from the pattern found in starving leaves incubated aerobically in a wilting or non-wilting condition (Macpherson, 1952*a*, *b*).

Table 3 shows that, besides α-amino nitrogen, changes in the peptide, volatile, amide and residual or unaccounted non-protein-nitrogen fractions occur in incubated juice. These changes may place limitations on the use of α-amino nitrogen as a measure of protein breakdown. The increase in ammonia concentration has been found in all clover-juice samples examined whether chloroform toluene or both chloroform and toluene are used as bactericides. The absence of bacteria in the media has been demonstrated by plating methods. The formation of ammonia has, in a number of cases, been accompanied by a fall in the amide content, but in no case has this fall amounted to more than a third of the ammonia formed. At least part of the ammonia increment may represent a loss of α-amino nitrogen by deamination.

The 'unaccounted non-protein nitrogen' represents the non α-amino nitrogen of lysine, arginine

Table 3. *Nitrogen redistribution in clover leaves ensiled over chloroform and in clover juice incubated over chloroform*

Incubation temperature, 30°. 'Unaccounted non-protein N' represents soluble non-protein N less the total of amide, steam-volatile, α-amino and peptide N. As this estimate is based on the α-amino N content after hydrolysis (i.e. the sum of α-amino and peptide N) it is free from any ambiguity about the conversion factor from the chelated-copper determination (see text). L.S.D. (5% and 1%), least significant differences at 5% and 1% levels.

Treatment		Total N (% of dry matter)	Nitrogen (% of total N)							
			Soluble	Soluble non-protein N	α-Amino	Peptide	Steam-volatile	Ammonia	Amide	Unaccounted non-protein N
Ensiled	0 hr.	5·4	28·0	28·3	4·4	9·7	1·1	0·6	3·7	9·0
	2 hr.	5·3	37·8	38·0	5·9	11·4	1·1	0·6	4·4	15·2
	24 hr.	5·2	63·6	63·0	20·8	10·7	1·7	1·6	4·2	25·6
	48 hr.	5·2	76·7	75·9	26·0	15·1	2·2	1·8	4·9	27·7
		(mg./ml.)								
Juice	0 hr.	5·0	27·9	27·5	6·8	7·1	1·0	0·6	4·4	8·2
	4 hr.	4·7	49·6	49·3	14·6	8·8	1·3	0·8	5·3	19·3
	24 hr.	4·7	63·8	64·1	22·9	10·0	2·6	2·2	5·4	23·2
	48 hr.	4·8	64·7	64·9	23·8	11·1	3·1	2·6	5·0	21·9
L.S.D. (5%)			2·6	2·7	0·9	1·4	0·2	0·2	0·7	—
L.S.D. (1%)			3·6	3·8	1·4	1·9	0·3	0·3	1·0	—

Table 4. *Ninhydrin-reacting substances found on two-dimensional chromatograms of incubation media*

Chromatograms were on Whatman no. 1 paper, with butanol–acetic acid–water (4:1:5) and water-saturated phenol, pH 5·5, as solvents. Figures represent increasing intensity of the spots produced. In interpreting the figures due allowance must, of course, be made for the fact that some amino acids, e.g. glutamic acid, give very intense, and some, e.g. histidine, relatively weak spots with ninhydrin. System A, crude enzyme and endogenous substrate; system B, crude enzyme and added gelatin; system C, partially purified enzyme and added gelatin. rt., Trace.

	System A			System B			System C		
Incubation time (hr.) ...	0	24	48	0	24	48	0	24	48
Ninhydrin-reacting substance									
Alanine	1	6	7	½	6	8	tr.	6	10
γ-Aminobutyric acid	2	2	2	tr.	tr.	tr.	0	0	0
Arginine	2	4	5	tr.	2	4	0	3	5
Asparagine	4	4	3	½	½	½	0	0	0
Aspartic acid	3	4	5	½	3	4	½	3	4
Glutamic acid	4	6	6	1	3	4	½	3	5
Glutamine	2	1	0	tr.	0	0	0	0	0
Glycine	0	2	2	0	2	4	0	2	4
Histidine	0	1	1	0	0	tr.	0	0	tr.
Leucines	1	6	6	0	1	3	tr.	3	5
Lysine	1	3	3	0	0	1	0	0	1
Methionine	tr.	1	1	0	0	1	0	1	2
Phenylalanine	1	2	2	0	0	1	0	0	1
Proline	0	2	2	0	1	2	0	1	3
Serine	3	5	5	½	2	4	tr.	2	3
Threonine	tr.	3	3	tr.	1	2	tr.	1	2
Tyrosine	tr.	2	2	0	0	tr.	0	tr.	1
Valine	1	3	3	tr.	1	2	tr.	2	4

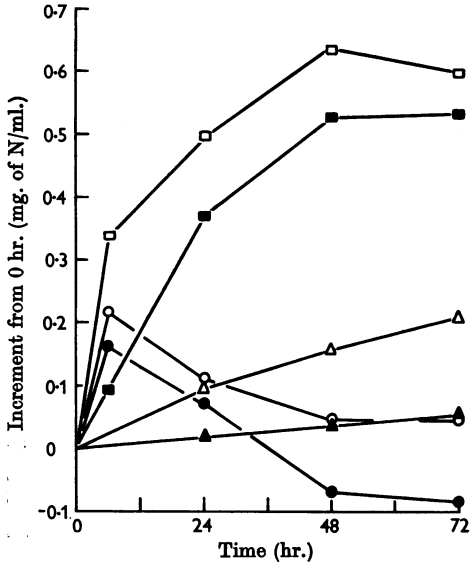

Fig. 1. Changes in nitrogen distribution in a sample of clover juice incubated at 25° and 50°: □, α-amino nitrogen, 50°; ○, peptide nitrogen, 50°; △, ammonia nitrogen, 50°; ■, α-amino nitrogen, 25°; ●, peptide nitrogen, 25°; ▲, ammonia nitrogen, 25°.

and histidine, hydrolysed nucleic acids and other, largely unknown, fractions. Decarboxylation of glutamic acid to γ-aminobutyric acid would transfer nitrogen from the α-amino nitrogen to the 'unaccounted non-protein nitrogen' columns of Table 3. This reaction has been reported as quantitatively important in the early hours of ensilage of grass (Macpherson & Slater, 1959). Examination of ethanolic extracts of ensiled clover leaves or of autolysing clover juice by two-dimensional paper chromatography have not, however, revealed any significant increases in the amounts of γ-aminobutyric acid present. Nor, when 0·05 M-glutamic acid was added to clover juice or the concentrated enzyme, could any γ-aminobutyrate formation be detected chromatographically during incubation under the assay conditions. The chromatographic evidence (Table 4) indicates that no ninhydrin-detectable substances, not estimated as α-amino nitrogen, were formed in any quantity during the assays on either endogenous or gelatin substrate.

Fig. 1 shows the changes in the concentrations of amino, peptide and ammonia nitrogen in a sample of clover juice incubated at 25° and 50°. At each temperature peptides accumulated during the initial stages of the reaction; subsequently most of the accumulated peptides were hydrolysed (in fact at 25° this phase continued till there was an apparent net loss of peptide nitrogen).

Since, in the initial stages of the reaction, peptides are obviously a large proportion of the molecules bound to the copper of the Pope & Stevens (1939) reagent, the factor of two is here particularly inappropriate for estimates of α-amino nitrogen before hydrolysis, and the quantity of the

latter is overestimated; consequently bound α-amino nitrogen is underestimated. As the reaction proceeds these errors will decrease. These considerations indicate a rate of release of free α-amino nitrogen which is slower than that shown in Fig. 1, but remains at a relatively high rate for a longer period. When chloramine-T was used to measure α-amino nitrogen, the reaction appeared linear with respect to time over a longer period. Consequently, the ratio of activity (chloramine-T) to activity (copper-chelating) steadily increased, a result explicable in terms of the greater reactivity of the Pope & Stevens reagent with peptide nitrogen.

Fig. 2 shows the changing concentrations of α-amino, peptide and ammonia nitrogen when the concentrated enzyme acts on a gelatin substrate.

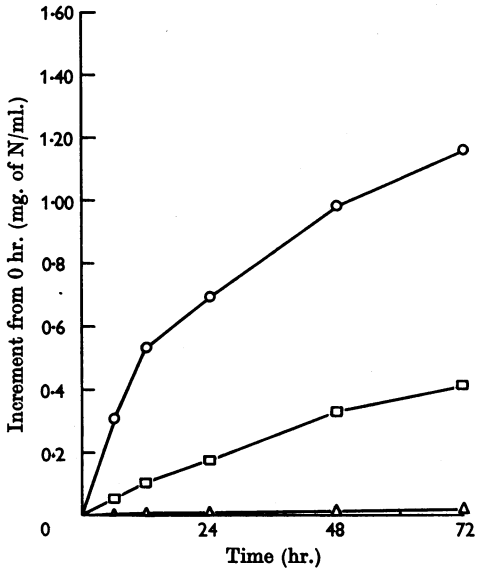

Fig. 2. Changes in nitrogen distribution when concentrated clover protease is incubated with gelatin at 45°. Clover nitrogen, 0·178 mg./ml.; gelatin, 24 mg./ml.; phosphate, 0·05 M, pH 6·0. □, α-Amino nitrogen; ○, peptide nitrogen; △, ammonia nitrogen.

This system is characterized by (1) a very large accumulation of peptide nitrogen, (2) an extended reaction time before equilibrium is reached, and (3) a negligible release of ammonia. The equilibrium position varies with temperature, and at 45° corresponds to about a 40 % splitting of the peptide bonds.

A comparison of Figs. 1 and 2 indicates the possibility that much of the peptidase function of the protease complex has been lost during concentration. However, allowing the crude enzyme to act on gelatin under these assay conditions gave results essentially similar to those shown in Fig. 2, with the single exception that a little more ammonia was released. The accumulation of peptides seems then to be a function of the substrate or of substrate concentration, and not due to changed properties of the enzyme.

Variations between juice samples. Juice from individual, glasshouse-grown clover plants was tested for protease activity on endogenous substrate with a group of six plants on each of a number of harvest dates. The results, shown in Table 5, indicate wide variations both between plants and between harvest dates. Protease activity was measured in two clones, each of eight plants, derived from plants which had shown very high and very low activity when initially tested; no differences between clones could be demonstrated. Environmental effects obviously influence protease activity on endogenous substrate.

Table 5. *Protease activity in groups of six plants at six harvest dates*

Harvest date	Protease (unit/mg. of protein N) Mean	5 % confidence interval
23. ix. 58	0·199	±0·026
30. ix. 58	0·221	±0·031
16. x. 58	0·144	±0·044
21. x. 58	0·173	±0·041
9. i. 59	0·154	±0·072
11. i. 59	0·209	±0·061

Table 6. *Protease and ammonia-producing activity in three clover samples assayed in three systems*

Assay system A, crude enzyme and endogenous substrate; assay system B, crude enzyme and gelatin substrate; assay system C, partially purified enzyme and gelatin substrate.

Incubation time (hr.)	Assay system A			Assay system B			Assay system C		
	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3	Sample 1	Sample 2	Sample 3
	Increment in α-amino N/mg. of protein N								
24	0·285	0·255	0·309	0·464	0·601	0·732	2·145	2·162	2·204
48	0·354	0·286	0·344	0·713	0·936	0·997	3·362	3·349	3·382
	Increment in ammonia N/mg. of protein N								
24	0·025	0·029	0·024	0·012	0·016	0·018	Nil	Nil	Nil
48	0·051	0·072	0·037	0·033	0·032	0·035	Nil	0·044	0·013

Juice, from clover harvested in each of three non-adjacent areas (1 yard square) in a field plot, was tested at pH 6·0 for the increment in α-amino and ammonia nitrogen when incubated at 45° in each of the three systems: (1) juice alone, (2) crude enzyme and gelatin substrate and (3) concentrated enzyme (schedule B, Table 2) and gelatin substrate. The results, shown in Table 6, indicate that the differences between samples apparent when testing crude enzyme on endogenous substrate, and in the presence of gelatin, disappear when the protease is partially purified. Since the yield of enzyme was similar from each sample, juice factors appear to influence considerably the activity of the crude enzyme.

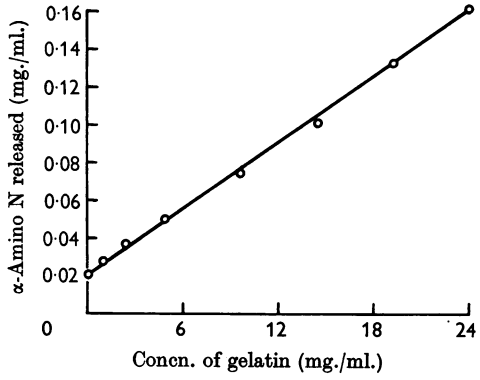

Fig. 3. Variation of activity with substrate concentration. Unfractionated juice was used as enzyme source, and an incubation time of 5 hr.; clover nitrogen, 0·65 mg./ml.; phosphate, 0·02M; sodium thioglycollate, 0·02M; pH 6·0; temperature 45°.

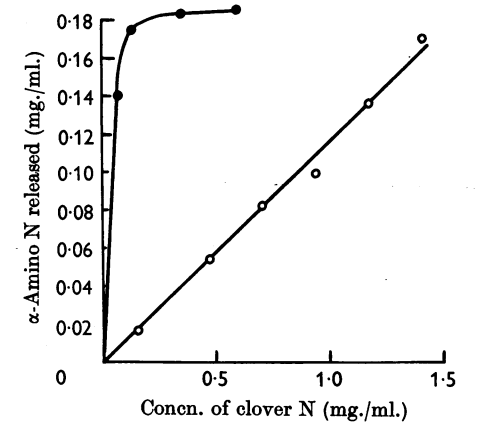

Fig. 4. Variation of activity with enzyme concentration. Incubation time was 16 hr. at 25°; gelatin concn., 24 mg./ml.; phosphate, 0·05M; sodium thioglycollate, 0·02M; pH 6·0. ●, Concentrated enzyme; ○, crude enzyme.

Properties of the assay system

Effect of substrate concentration. In Fig. 3 is shown the effect of variation in gelatin concentration on the protease activity of the crude enzyme over an incubation period of 5 hr. The linear response to increased gelatin concentration, together with an increase in α-amino nitrogen in the control, suggests that hydrolysis of endogenous substrate continues in the presence of gelatin.

When an incubation time of 24 hr. was used, and higher concentrations of gelatin were examined, there was a markedly decreased response to concentrations in excess of 25 mg./ml. and half-maximum response occurred at about 16 mg./ml.

No detailed survey has been carried out on the activity of the clover enzyme on a range of protein substrates. Casein (dissolved with heat at alkaline pH) was hydrolysed at pH 6·0 and 6·5, though the rate of release of α-amino nitrogen appeared to be lower than from gelatin at the same concentration.

Effect of enzyme concentration. The relative activities of the two types of protease preparation as a function of enzyme concentration, in a gelatin medium, are demonstrated in Fig. 4. In this system, the crude preparation gave a response proportional to clover nitrogen, over all of the range that could be conveniently tested.

pH *effects.* The effect of pH on the activity of the crude enzyme, with a gelatin substrate, is shown in Fig. 5. The optimum pH lay in the range 5·9–6·3, and activity decreased sharply on the acid side of this range, less sharply on the alkaline side. Potassium acetate–acetic acid buffers (0·1M) were used below pH 5·0, and disodium monohydrogen phosphate–potassium dihydrogen phosphate (0·05M) buffers at higher pH values. Closer investigation of the limiting pH showed no activity at pH 3·9, or at any lower pH (1·0–3·8) investigated. No observations were made at values more alkaline than pH 7·8. The concentrated enzyme showed the same optimum and limiting pH as the unfractionated juice.

The effect of pH on stability was measured on samples of crude enzyme, held for 16 hr. at 4° after pH adjustment with lactic acid or potassium hydroxide, and then assayed on a gelatin substrate at pH 6·0. Thioglycollate, 0·02M, was present during storage. At pH 6·5 and above, the pH dropped by 0·2–0·3 unit during storage, and in Fig. 6, which relates residual activity to storage pH, the pH value at the end of storage has been used. Two separate clover samples each retained 60–70% of their original activity after storage treatment at pH 5·5–7·0. Outside this range increasing acidity caused a marked decrease in residual activity and increasing alkalinity had a comparatively small effect. The concentrated protease was tested for

stability when held for 2 hr. at 45°. The preparation retained 75–85 % of its activity when kept in the range pH 5·0–7·0, 65 % at pH 8·0 and 35 % at pH 9·0. In another experiment a sample of the concentrated enzyme was adjusted to pH 4·5 and stored at 30°. This preparation retained 90 % of its activity after 4 hr., 80 % after 24 hr. and 65 % after 30 hr. The stability of the concentrated enzyme appeared to be much greater than that of the unfractionated juice.

Temperature effects. The sensitivity of the protease to temperature inactivation was measured on samples of crude enzyme at pH 6·0. These were heated at various temperatures for 5 min. in the presence of 0·02 M-thioglycollate, cooled rapidly and then assayed for residual activity by incubation for 5 hr. at 45°. The results obtained, with two samples, are shown in Fig. 7. The effect of incubation temperature over a period of 3 hr. on the activity on endogenous substrate of two juice samples containing thioglycollate is shown in Fig. 8. As the incubation period was extended, the differences between 45° and lower temperatures did, of course, become less marked. None the less, with an incubation period of 24 hr., activity at 45°

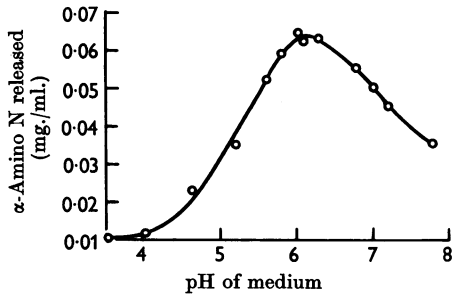

Fig. 5. Protease activity at various pH values. Unfractionated juice was used as enzyme source. Activity is expressed as the increase in the α-amino-nitrogen concentration during 5 hr. incubation at 45°; phosphate (0·05 M) or acetate (0·1 M); sodium thioglycollate, 0·02 M; gelatin, 20 mg./ml.; clover protein, 0·74 mg./ml.

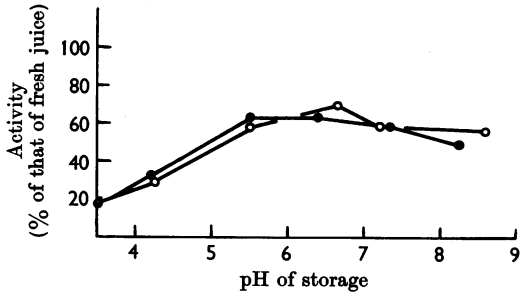

Fig. 6. Effect of pH on the stability of the protease of two samples of unfractionated clover juice stored for 16 hr. at 4°. Activity was measured during incubation for 5 hr. at 45°, and is expressed as a percentage of the activity of a sample not stored. Sodium thioglycollate (0·02 M) was present during storage.

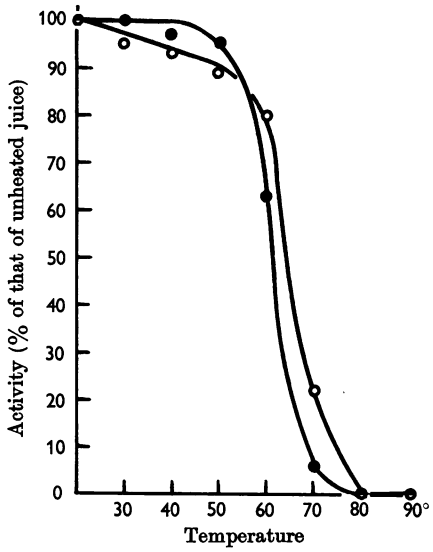

Fig. 7. Effect of heat on the protease activity of two samples of crude enzyme. Residual protease activity was measured after heating for 5 min. at various temperatures in the presence of 0·02 M-sodium thioglycollate. Incubation was with gelatin (20 mg./ml.), phosphate, 0·05 M, pH 6·0, for 5 hr. at 45°.

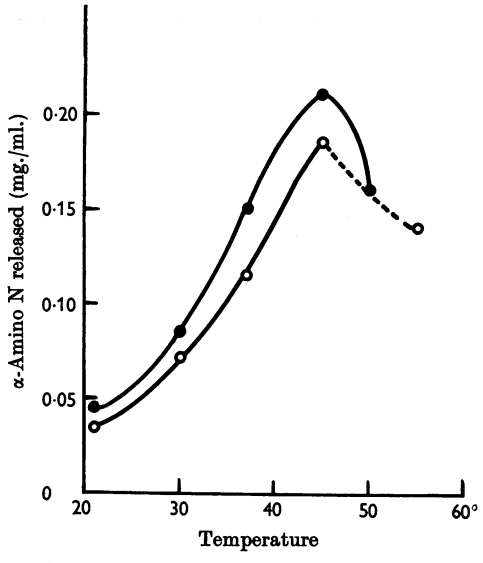

Fig. 8. Effect of incubation temperature on protease activity of two samples of crude enzyme acting on endogenous substrate. Incubation was for 3 hr.; phosphate, 0·05 M; sodium thioglycollate, 0·02 M; pH 6·0.

was still greater than that at 38°. Similar results were obtained with crude enzyme in the presence of gelatin.

Activators and inhibitors

Bactericides. In comparative assays no difference was found in the proteolytic activity in the presence of toluene or chloroform. With thymol, a slightly lower activity was recorded. The rate of release of α-amino nitrogen in clover-juice samples incubated for 3 hr. or 6 hr. with and without chloroform was the same.

Reducing agents. When sodium bisulphite and sodium thioglycollate were added to the crude enzyme to give a final concentration of 0·02 M, the amount of proteolysis during incubation for 24 hr. at 45° was increased by 35–40 %. When a gelatin substrate (24 mg./ml.) was added to the crude enzyme, bisulphite and thioglycollate had no effect on the proteolysis. Similarly, additions of reducing agents had no activating effect on the concentrated clover enzyme acting on the gelatin substrate for 24 hr. at 45°. In subsequent experiments no thioglycollate was added to the concentrated enzyme system.

Other agents. The activity of the concentrated enzyme–gelatin system in the presence of a number of possible inhibitors is shown in Table 7. The effect of concentration on the action of some of these is shown in more detail in Fig. 9. All these results refer to the system acting for 24 hr. at 45° and pH 6·0. A number of the substances interfered with the Pope & Stevens method of analysis. In the presence of cysteine, potassium cyanide and manganous sulphate, the chloramine-T method was used to estimate α-amino nitrogen. When additions of hydroxylamine or hydrazine were made proteolysis was measured (*a*) by the decrease in the amount of nitrogen precipitated by cold 5 % trichloroacetic acid, and (*b*) by the increase in the amount of α-amino nitrogen eluted by 0·8 N-hydrochloric acid from columns of Dowex-50 (nominal 8 % cross-linked; 200–400 mesh).

The results in Table 7 illustrate the lack of response of the system to reducing agents, and the comparatively slight action of oxidizing agents. At a concentration of 0·2 M, cysteine and sodium bisulphite were strongly inhibitory and sodium thioglycollate caused 35 % inhibition. Metal-binding agents and heavy-metal ions were, in general, strongly inhibitory. A feature of the inhibition by a number of agents was partial inhibition over a considerable concentration range (Fig. 9). This may be indicative of the complexity of the enzyme mixture assayed.

When activity was measured by the increase in the 'chloramine-T amino nitrogen', manganous sulphate at a concentration of 0·02 mM showed a stimulatory effect which, though variable, amounted at times to as much as 25 %. Lower concentrations were without effect, and higher concentrations (0·1 mM) were inhibitory. This concentration of manganous sulphate (0·02 mM) had no serious effect on the Pope & Stevens estimates, but assay of the enzyme system by this method in no

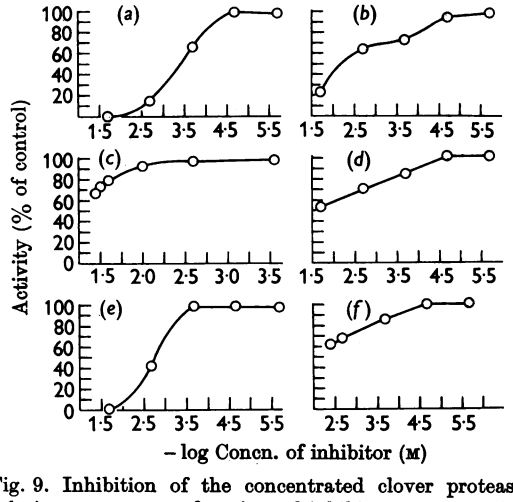

Fig. 9. Inhibition of the concentrated clover protease–gelatin system as a function of inhibitor concentration. Incubation was for 24 hr. at 45° and pH 6·0. (*a*) Mercury ion; (*b*) zinc ion; (*c*) iodoacetic acid; (*d*) ethylenediaminetetra-acetic acid; (*e*) 8-hydroxyquinoline; (*f*) *p*-chloromercuribenzoate.

Table 7. *Effect of a number of potential activators or inhibitors on the concentrated clover protease–gelatin system*

Activity was measured over 24 hr. at 45°. Each treatment had four replicates. Agreement between replicates varied between experiments, but mean differences of 5 % were always significant.

	Activity (% of control)		
Concn. of activator or inhibitor (mM)	20	2	0·2
Addition			
Sodium thioglycollate	102	103	—
Sodium bisulphite	103	101	—
Cysteine	71	95	—
Potassium cyanide	0	71	90
Hydrogen peroxide	90	100	—
Hydroxylamine	100	100	—
Hydrazine	100	102	—
Iodoacetic acid	70	97	97
8-Hydroxyquinoline	0	42	98
p-Chloromercuribenzoate	—	68	85
Ethylenediaminetetra-acetate	53	71	84
Mercuric sulphate	0	15	66
Zinc sulphate	23	64	72
Manganous sulphate	0	64	86
Silver nitrate	20	—	—
Cadmium chloride	17	—	—
Cupric chloride	5	—	—

case revealed stimulation due to manganese. The Pope & Stevens reagent is more reactive with peptides than is chloramine-T. The sensitivity of the chloramine-T method is greater when small peptides are hydrolysed to amino acids. The fact that a chloramine-T assay, but not a Pope & Stevens assay, reveals stimulation due to 0·02 mM-manganous sulphate may indicate that the manganous ion stimulates the di- or tri-peptidase function of the proteolytic system.

Most of these observations have been confined to the concentrated enzyme–gelatin system. The effects of 0·02 M-mercuric chloride, and of potassium cyanide and hydroxylamine, have been confirmed on crude enzyme with endogenous substrate.

DISCUSSION

Use of the α-amino-nitrogen increment as a measure of proteolysis in these preparations has a number of limitations, not all of which can be satisfactorily evaluated: (1) Proteolysis may be underestimated in juice samples by reason of deamination reactions. (2) Peptide-bond hydrolysis will be underestimated in those circumstances where peptide accumulation is considerable. In this regard, the copper-chelation method, which has considerable reactivity with peptides, has some advantages (as a measure of protein hydrolysis) over the more specific α-amino-nitrogen methods. (3) The factors causing the large increment in 'residual non-protein nitrogen' in juice samples are largely unknown.

Separate samples of the crude clover enzyme, although showing large variations in activity, have differed little in response to changes in such environmental factors as temperature and pH. Such variations as have occurred (Figs. 6–8) may well be due to experimental error. Thus the white-clover enzyme appears to behave consistently in relation to the temperature and pH of the environment, and the data presented can be considered to apply generally within this plant species. Although information gained about optimum conditions for a gelatin substrate cannot be automatically applied to action on the plant substrate, when action on endogenous and gelatin substrates was compared very similar responses were obtained. Again the data presented about limiting conditions (pH and temperature stability) appear to apply equally to each substrate.

Substrate differences were apparent in that action on the endogenous substrate but not on gelatin was greater in the presence of reducing agents. No evidence is available on the reasons for this difference, but it may be related to the lack of thiol groups in the gelatin molecule. The activity of many plant proteases is greater in the presence of reducing agents (Greenberg & Winnick, 1945), and Tracey (1948) found that the tobacco-leaf protease tested on a gelatin substrate was activated by a number of different reducing agents. The influence of sodium bisulphite on enzyme activity is interesting in relation to the use of sodium metabisulphite as a preservative during ensilage.

No evidence has been gained on the complexity of the leaf-protease system. The assumption is made that the concentrated enzyme retains the complete system as found in the crude enzyme preparation. The evidence for this rests on the similarities of the properties and the reactions of the two preparations.

The optimum pH, 5·9–6·3, is higher than that reported by Tracey (1948) for the tobacco enzyme, but within the range pH 5–7, which is generally found to be optimum for plant proteases acting on protein substrates. The clover enzyme also appeared to differ from the tobacco protease in its greater sensitivity to acid, and higher activity at slightly alkaline pH values.

Tracey (1948) noted variation in the proteolytic activity of a number of species, and Holden & Tracey (1948*a*, *b*) described differences due to fertilizer treatment and virus infection. In terms of Tracey's protease unit, the clover-juice activity ranged from 60 to 250. This is in the lower part of the range of 25–1040 noted by that author, and somewhat below his modal observation, which appeared to be from 250 to 400.

The pH and temperature conditions which limit the activity of the enzyme are important to an understanding of the extent of proteolysis occurring during ensilage. If we assume that we can apply the data from 'crude-enzyme' studies to the starving leaf, the absolute limit to activity lies just below pH 4·0, a value which, if reached at all, would only be reached after several days of ensilage. By this time, the enzyme reaction might have proceeded to equilibrium. The results reported in Fig. 6, however, indicate that at low pH values the enzyme becomes increasingly unstable. Enzyme inactivation may then occur before enzyme activity is finally halted by limiting pH. The temperature stability of the enzyme is such that temperature inactivation during ensilage is probably not important. High temperatures are, in any case, undesirable because of their effect on protein digestibility (Watson & Ferguson, 1937). The temperature régime, most suitable for the limitation of protein breakdown, will depend upon the interaction of the temperature coefficient of protease inactivation and the temperature effects on proteolysis and acid production. Since much of the proteolysis occurs in the first 24 hr., and in most cases, even at relatively low temperatures, equilibrium is reached within 48 hr., the difficulties

of limiting proteolysis by spontaneous fermentation are apparent.

In this regard, the relatively large differences between different samples of clover juice may also be considered. If, in most silos, the proteolytic reaction will proceed to equilibrium before limiting conditions or enzyme inactivation halt it, then samples which have an equilibrium corresponding with a small percentage of solubilized nitrogen rather than samples having a slow rate of reaction are most desirable. Not all samples examined that showed low activity under the standard assay conditions (24 hr. at 45°) have given an equilibrium indicating slight proteolysis when incubation has been extended. Indeed, there has been a tendency for the samples of low activity to have a relatively high ratio of α-amino nitrogen to total nitrogen in the untreated juice and to reach equilibrium at about the same point as the samples of high activity (i.e. in terms of the ratio of α-amino nitrogen to total nitrogen). However, relatively few samples have been examined in this detail, and not all of them have corresponded to this pattern. Samples of low activity and an equilibrium with low hydrolysis have been encountered. Obviously, further observations will be necessary before this matter can be fully appreciated.

SUMMARY

1. Nitrogen catabolism in sap expressed from leaves of *Trifolium repens* has been studied, and the use of the increment in α-amino content as an index of protein hydrolysis in the leaves is discussed.

2. A considerable variation in the rate of release of α-amino nitrogen in different samples of sap was found.

3. A fraction showing about five times the protease activity of fresh sap has been prepared by removal of relatively inactive protein.

4. Some characteristics of the hydrolysis of endogenous protein and gelatin by the crude enzyme, and of gelatin by the concentrated enzyme, were examined.

5. With endogenous substrate, peptides accumulated briefly in the medium, then rapidly disappeared. With relatively high concentrations of gelatin substrate there was a marked accumulation of peptides in the medium.

6. The protease showed a broad pH optimum of 5·9–6·3; no activity could be detected at values lower than pH 4·0. The crude enzyme was rapidly inactivated at pH values below 5·0, but stability was not affected by pH values between 5·0 and 7·0. In the concentrated condition the enzyme was much more stable on storage.

7. Reducing agents enhanced activity on endogenous substrate but not on gelatin.

8. Chelating agents and heavy-metal ions were the most active of a number of possible inhibitors examined.

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