Beiträge zur Tabakforschung International · Volume 12 · No. 4 · July 1984 DOI: 10.2478/cttr-2013-0543

Partial Purification and Characterization of Proteases in Tobacco Leaf and Callus*

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SUMMARY

Ammonium sulfate fractionation, gel permeation and cation-exchange column chromatography were employed for partial purification of proteases from leaf laminae and callus tissues of Samsun NN tobacco (*Nicotiana tabacum* L.). The predominant proteases in the leaf and callus are acidic sulfhydryl proteases which are activated by 2-mercaptoethanol and ethylenediamine tetraacetic acid, completely inhibited by iodoacetic acid, and partially inhibited by phenylmethane sulfonyl fluoride and pepstatin A. With hemoglobin and tobacco Fraction I protein as substrates, leaf and callus proteases showed a pH optimum of 5. However, specific activity was significantly higher in the callus than in the leaf. Tobacco proteases digested hemoglobin more effectively than Fraction I protein and showed the least activity with casein. Gel permeation resolved three protease variants in leaf extracts but only two in callus samples. Rechromatography of the large molecular weight fraction in a cation-exchange column produced three and two variants for leaf and callus, respectively. The present results suggest that there are at least five variants of sulfhydryl protease in tobacco leaf and three in callus tissue and that tobacco Fraction I protein can be metabolized by both leaf and callus proteases.

ZUSAMMENFASSUNG

Zur partiellen Reinigung von Proteasen aus Blatt- und Kallusgewebe von Samsun-NN-Tabak (*Nicotiana tabacum* L.) wurden fraktionierte Ammoniumsulfatfällung, Gelchromatographie und Kationenaustauschchromatographie eingesetzt. Die Proteasen in Blatt und Kallus von Tabakpflanzen sind hauptsächlich saure Sulfhydrylproteasen, die durch 2-Mercaptoethanol und Ethylendiamintetraessigsäure aktiviert und durch Jodessigsäure vollständig und Phenylmethansulfonylfluorid und Pepstatin A teilweise inhibiert werden. Bei Verwendung von Hämoglobin und Fraktion-I-Protein als Substrate hatten die Blatt- und Kallusproteasen ein pH-Optimum von 5. Die spezifische Aktivität war im Kallusgewebe jedoch signifikant höher als im Blattgewebe. Die Tabakproteasen bauten Hämoglobin stärker ab als Fraktion-I-Protein und zeigten mit Casein die geringste Aktivität. Durch Gelchromatographie wurden im Blattextrakt drei und im Kallusextrakt nur zwei Proteasevarianten getrennt. Die Rechromatographie der hochmolekularen Fraktion an einem Kationenaustauscher ergab für das Blattgewebe drei und für das Kallusgewebe zwei Proteasevarianten. Die vorliegenden Ergebnisse deuten darauf hin, daß im Blattgewebe der Tabakpflanze mindestens fünf und im Kallusgewebe mindestens drei Varianten der Sulfhydrylprotease vorkommen und daß das Fraktion-I-Protein des Tabaks sowohl durch Blatt- als auch durch Kallusproteasen abgebaut werden kann.

RÉSUMÉ

Afin de purifier partiellement de ses protéases le tissu foliaire et calleux du tabac Samsun-NN (*Nicotiana tabacum* L.), on a procédé à une précipitation fractionnée par le sulfate d'aluminium, à une chromatographie sur gel et une chromatographie par échange cationique. Les protéases contenues dans la feuille et le callus des plantes de tabac sont, pour l'essentiel, des protéases acides sulfhydriques lesquelles sont activées par le 2-mercaptoéthanol et l'acide éthylènediaminetétraacétique, entièrement inhibées par l'acide iodoacétique et partiellement inhibées par le fluorure de sulfonylphénylméthane et la pepstatine A. L'utilisation d'hémoglobine et de protéine fraction I en tant que substrat, avait permis aux protéases de la feuille et du callus d'atteindre un pH optimum de 5. Toutefois, au sein du tissu

* Received: 26th January 1983 — accepted: 9th November 1983.

calleux, l'activité spécifique était nettement supérieure à celle du tissu de la feuille, et cela de manière significative. Les protéases du tabac digérèrent plus efficacement l'hémoglobine que la protéine fraction I et firent preuve de la plus faible activité sur la caséine. La chromatographie sur gel permit la séparation de trois variantes de protéases dans les extraits de feuilles et de deux seulement dans les échantillons de callus. La rechromatographie de la fraction à haut poids moléculaire sur échangeur cationique, fit apparaître pour le tissu de la feuille trois variantes de protéases et deux pour celui du callus. Les résultats ainsi obtenus indiquent que le tissu de la feuille de tabac présente au moins cinq variantes de la protéase sulfhydrique alors que le tissu du callus en contient au moins trois et que la fraction I des protéines du tabac est détruite tant par les protéases de la feuille que par celles du callus.

INTRODUCTION

Plant leaves contain proteases capable of digesting animal proteins such as casein (5) or hemoglobin (3, 6) and plant protein such as Fraction I protein (12, 17). Leaf proteases are important in protein turnover and in senescent processes in plants (13). Since tobacco has recently been advocated as a potential food crop for its soluble proteins, namely Fraction I protein and Fraction II protein (16), the activity of endogenous proteases could affect protein yield and quality. Knowledge about the characteristics of tobacco proteases is therefore needed. The most recent study of tobacco leaf proteases prior to the work in our laboratory was by *Anderson* and *Rowan* (1). In a recent study, we observed an enhancement of protease activity in tobacco cell suspension cultures supplied with tobacco Fraction I protein as the sole source of nitrogen (8). Whether proteases in cell culture resemble those in the leaf remains to be determined. An *in vitro* system would be advantageous in the elucidation of the genetic and epigenetic regulation of proteases in tobacco cells. The objective of this study is therefore to partially purify proteases from tobacco calli and to compare them with leaf proteases for some physical and chemical properties.

MATERIALS AND METHODS

Plant Materials

Tobacco plants *(Nicotiana tabacum* L. var. Samsun NN*) were grown in pots in the greenhouse during early spring with supplementary lights that provided a 14 h illumination. Fully expanded leaves were harvested and deveined before the flower bud stage. The leaf laminae were kept in the freezer (—70 °C) until extraction. Calli of the same tobacco variety were cultured on the Murashige-Skoog agar medium (11) containing 2,4—D (1 ppm) for 4 weeks. The callus mass was harvested and frozen immediately. The experiment was repeated once to ascertain the analytical results.

* A pair of dominant necrosis genes for Tobacco Mosaic Virus (TMV) resistance, i.e. Samsun NN is an isogenic line of Samsun for TMV resistance.

Extraction of Proteases

All extraction and purification procedures were carried out at 4 °C. Tobacco leaf laminae (150 g fresh weight) and calli (200 g fresh weight) were homogenized for 2 min with 300 to 400 ml of 50 mM potassium phosphate buffer (pH 7) containing 2 mM 2-mercaptoethanol in a Waring blender. The homogenate was filtered through four layers of cheesecloth and one layer of Miracloth and subsequently centrifuged at 12,000 g for 10 min. The filtrate was adjusted to 90 % saturation of $(NH_4)_2SO_4$ prior to centrifugation at 20,000 g for 30 min. The resulting pellet was dissolved in a small volume of 5 mM acetate buffer (pH 5) containing 2 mM 2-mercaptoethanol and dialyzed against the same buffer overnight unless otherwise stated.

Column Chromatography for Protease Fractionation and Molecular Weight Determination

The dialysate was loaded onto a Sephacryl S-200 column (3.5 × 32 cm) and pre-equilibrated with the acetate buffer mentioned above. The protein was eluted from the column with the equilibration buffer at a flow rate of 40 ml/h. The eluate was collected in 5 ml fractions and assayed for protein content and protease activity. A set of molecular weight series (blue dextran 2000, aldolase, ovalbumin, chymotrypsinogen A and ribonuclease A) was chromatographed in the same manner for estimation of the molecular weight of protease fractions. The major peak fractions of protease activity eluted from the Sephacryl S-200 column were pooled and loaded onto the same size column packed with SP-Sephadex C—50. The pre-equilibration and elution buffer was the same as that used in the Sephacryl S-200 chromatography. The column was first eluted with 200 ml of the buffer which was collected in 5 ml fractions. The bound protein was then eluted with 200 ml of the same buffer containing a linear gradient of 0 to 0.3 M NaCl at a flow rate of 30 ml/h. All fractions were assayed for protein content and protease activity.

Assay of Protease Activity

The standard reaction mixture (1.5 ml) contained an aliquot of dialysate or eluate (0.1 to 0.2 mg of protein), 0.1 ml of 2 % hemoglobin or Fraction I protein (18.4 mg/ml) extracted from tobacco leaf (9) and a volume of acetate buffer (pH 5) to give a final concentration of 50 mM. The reaction was carried out at 40 °C for 2 h and terminated by the addition of 0.6 ml of

16 % (w/v) trichloroacetic acid. The reaction mixture was subsequently filtered through a 0.2 μm microporous filter. The amount of acid soluble α-amino acids in the filtrate was determined by ninhydrin reaction (15). Protease activity was expressed as Δo.d.$_{570\,nm}$/mg protein/h.* The protein content of the protease preparations was determined by the method of *Lowry* et al. (10) using bovine serum albumin as a standard. For determination of protease activity as a function of pH, acetate buffer (pH 3 to 5), phosphate buffer (pH 6 to 8) and Tris-HCl buffer (pH 9 to 10) at 50 mM concentration were used in the reaction mixture.

Inhibitor Studies

According to *Barrett* (2), ethylenediamine tetraacetic acid, phenylmethane sulfonyl fluoride, iodoacetic acid and pepstatin A inhibit protease activity. Ethylenediamine tetraacetic acid (3 mM) and iodoacetic acid (5 mM) were dissolved in 50 mM acetate buffer (pH 5), whereas phenylmethane sulfonyl fluoride (3 mM) and pepstatin A (5 μM) were solubilized in absolute and 70 % ethanol, respectively. Inhibitor solution (0.1 ml) was added to the reaction mixture devoid of protein substrate. For experimental controls, the same volume of acetate buffer and ethanol was added instead. The mixture was pre-incubated at 40 °C for 30 min prior to the addition of protein substrate.

RESULTS

When hemoglobin was used as the substrate, protease activity was not detectable in the leaf extract prepared with phosphate buffer (pH 7) or acetate buffer (pH 5). The activity became measurable with an optimum at pH 6 if assayed with leaf protein precipitated in 90 % $(NH_4)_2SO_4$ saturation (Figure 1). Addition of 2-mercaptoethanol (2 mM) to the extraction buffer enhanced protease activity at all pH values and shifted the optimum to pH 5. The same protease preparation was studied for substrate specificity as a function of pH. Both hemoglobin and tobacco Fraction I protein had a pH optimum of 5 (Figure 2). However, the activity of Fraction I protein was only 43 % of that obtained with hemoglobin as the substrate. Casein appeared to be the poorest substrate among the three and was digested more under alkaline conditions. Because the specific activity of leaf protease was high and reproducible when assayed at pH 5 with 2-mercaptoethanol in the extraction buffer, subsequent studies were carried out under these conditions.

Fractionation of leaf protein on Sephacryl S-200 yielded three peaks, I_a, II_a and III_a, of protease activity when assayed with hemoglobin (Figure 3). The molecular weight of these peaks was estimated to be 0.5 to 1.5 × 10^5, 1 to 5 × 10^4 and 0.4 to 1 × 10^4 daltons, respectively. Peak I_a, which was the major fraction for protein content and protease activity, was fractionated on SP-Sephadex C-50 and separated into peaks I_b, II_b and III_b (Figure 4). Peaks I_b and II_b eluted with the equilibration buffer, whereas peak III_b eluted with 0.18 M NaCl. The ninhydrin reaction of the digestion mix-

Figure 1.
Protease activity as a function of pH in tobacco leaves extracted with or without 2-mercaptoethanol.

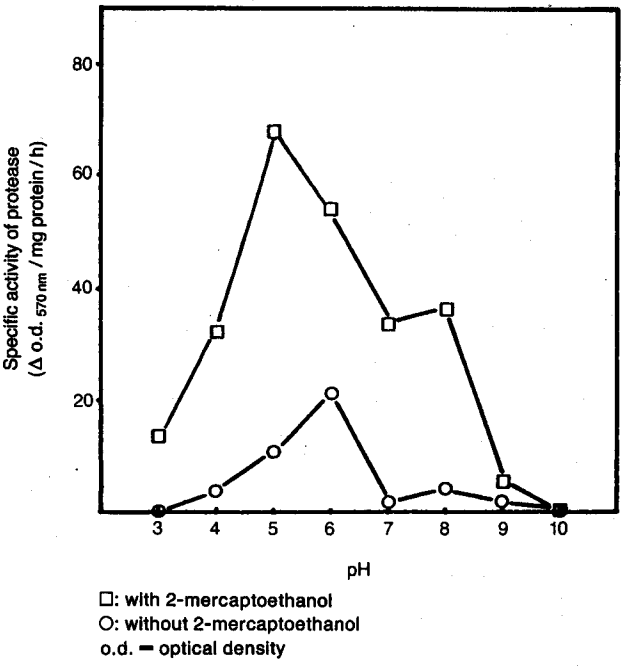
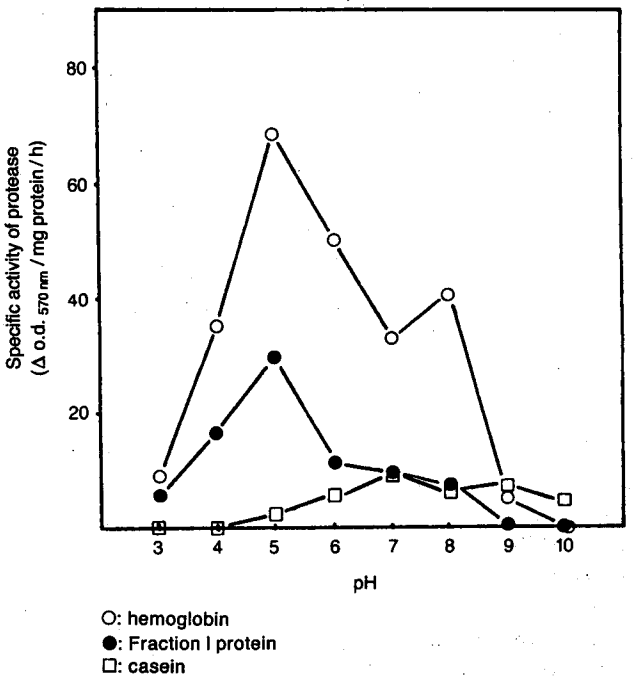


Figure 2.
Protein substrate specificity of tobacco leaf protease as a function of pH.



* o.d. = optical density.

Figure 3.
Sephacryl S-200 chromatography of protease from tobacco leaves.

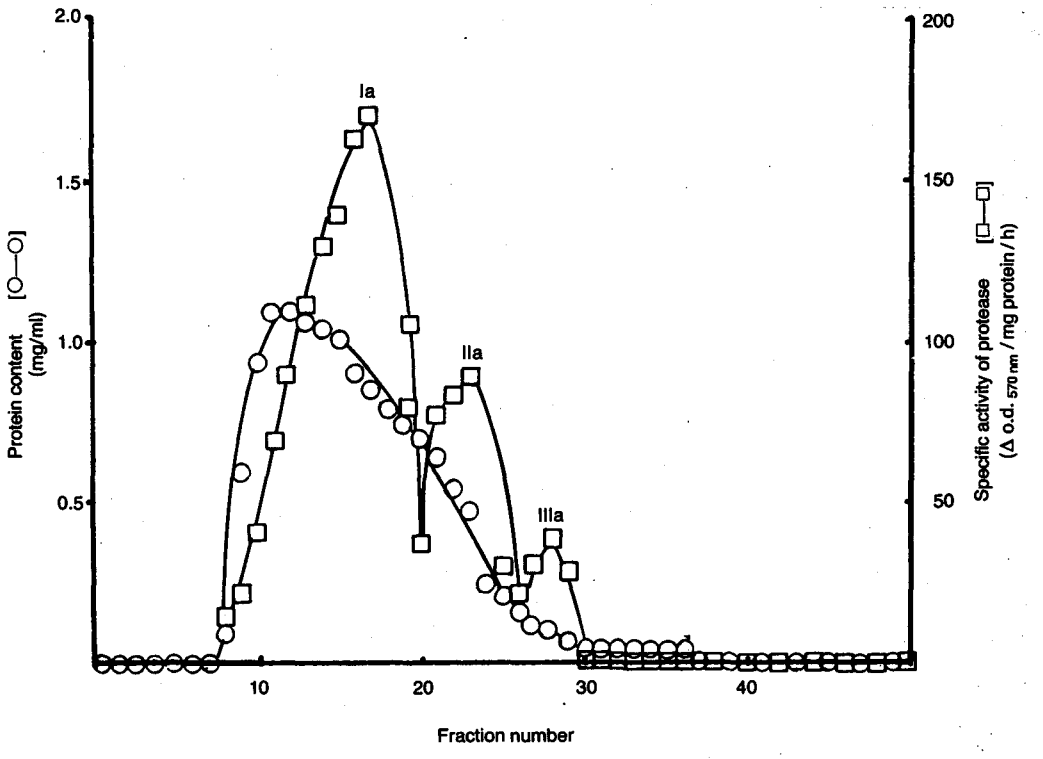
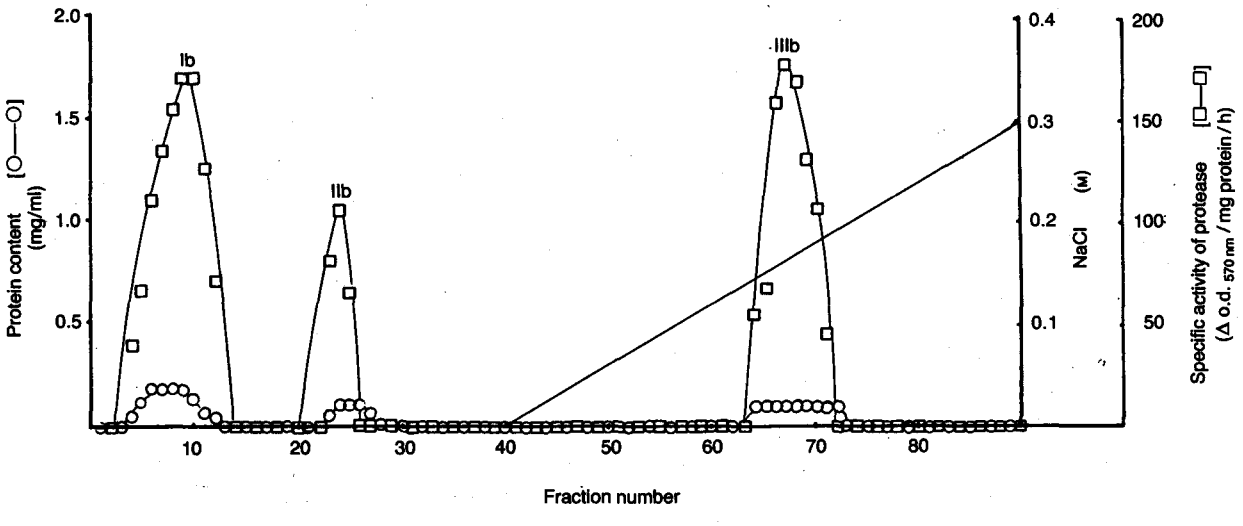


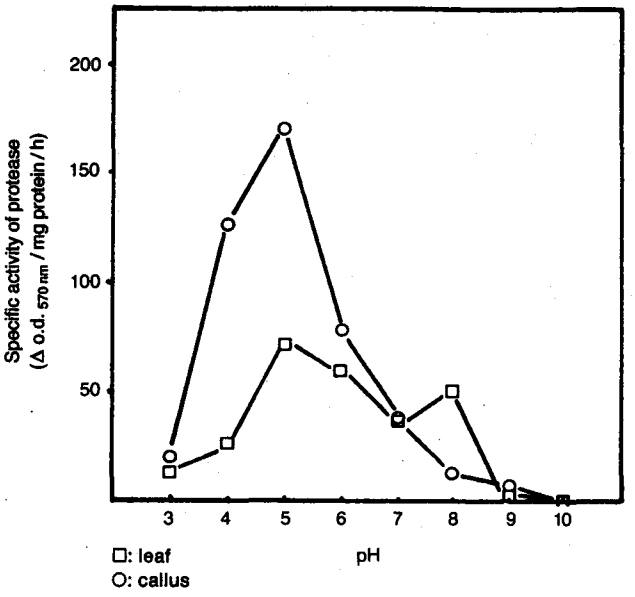
Figure 4.
SP-Sephadex chromatography of the I_a protease fraction from Sephacryl S-200 chromatography in tobacco leaves.



ture with proteases in peak II_b was yellow in color. A strong background coloration in ninhydrin reaction was observed when assaying the peak III_b fraction while peak I_b showed no abnormality in the ninhydrin reaction.

Proteases from callus extract which were precipitated in 90 % $(NH_4)_2SO_4$ solution exhibited the same pH 5 optimum as did the leaf proteases (Figure 5). The specific activity of callus proteases was twice that of leaf proteases at pH 4 and 5 but was similar at other pH values except pH 8 where leaf proteases had a higher activity. The catalytic efficiency of callus proteases for Fraction I protein digestion was about 30 % of that using hemoglobin as the substrate. This is in good agreement with the results from leaf proteases (Figure 2). Callus samples lacked peak III_a following gel perme-

Figure 5.
Comparison of the activities of proteases from tobacco leaves and calli as a function of pH.



ation (Figure 6). When peak I_a of callus proteases was refractioned on the cation-exchange column, it produced peaks I_b and III_b (Figure 7). Except for the absence of peaks III_a and II_b, the column chromatographic profiles of callus proteases are superimposable to those of leaf proteases. In all cases, the specific activity of protease fractions is higher in callus eluate than leaf eluate.

The results of partial purification of proteases from tobacco leaf and callus tissue are summarized in Table 1. A unit of protease activity is defined as the increase in optical density of 0.01 ml/h at 570 nm under the assay conditions described in Materials and Methods. Protease activity in the crude leaf extract was barely detectable, less than one-tenth of the value in the preparation of 90 % $(NH_4)_2SO_4$ saturation. This is probably indicative of protease inhibitors in the crude extract. A protein fraction of tobacco leaf extract with molecular weights of 1×10^3 to 1×10^4 inhibits proteases (*Xu* and *Sheen*, unpublished data). The protease units in callus extract and the corresponding $(NH_4)_2SO_4$ precipitate were comparable, suggesting a minimal interference of protease inhibitors. With the present scheme of partial purification, the major callus protease enhanced specific activity several-fold, but 54-fold for the leaf protease. The latter case is probably attributable to the removal of protease inhibitors during $(NH_4)_2SO_4$ precipitation.

Effects of chemical inhibitors on the activity of partially purified leaf proteases from cation-exchange chromatography are presented in Table 2. In comparison to the control, iodoacetic acid completely inhibited the activity of three protease fractions, whereas phenylmethane sulfonyl fluoride and pepstatin A showed partial inhibition. Of interest is the enhancement of activity by ethylenediamine tetraacetic acid in all cases although the degree of stimulation varied among the three fractions. Similar results were obtained with the I_b fraction of callus tissue.

Table 1. Partial purification of proteases from tobacco leaf and callus tissue.*

Purification step	Volume (ml)	Total activity (10^{-2} × units)	Total protein (mg)	Specific activity (units/mg)	Purification
Tobacco leaf laminae					
Homogenization with phosphate buffer (pH 7)	340	23.8	952	2.5	1
90 % $(NH_4)_2SO_4$ saturation	52	262.8	392	67.3	27
Peak I_a from Sephacryl S-200	35	221.5	177	125.4	50
Peak I_b from Sephadex C-50	30	99.8	73	136.1	54
Tobacco callus tissue					
Homogenization with phosphate buffer (pH 7)	500	185.3	250	74.1	1
90 % $(NH_4)_2SO_4$ saturation	50	192.0	114	168.4	2.3
Peak I_a from Sephacryl S-200	40	163.1	67	244.2	3.3
Peak I_b from Sephadex C-50	19	56.6	22	257.2	3.5

* The definition of protease unit is stated in the text.
Data are averages of two experiments.

Figure 6.
Sephacryl S-200 chromatography of protease from tobacco calli.

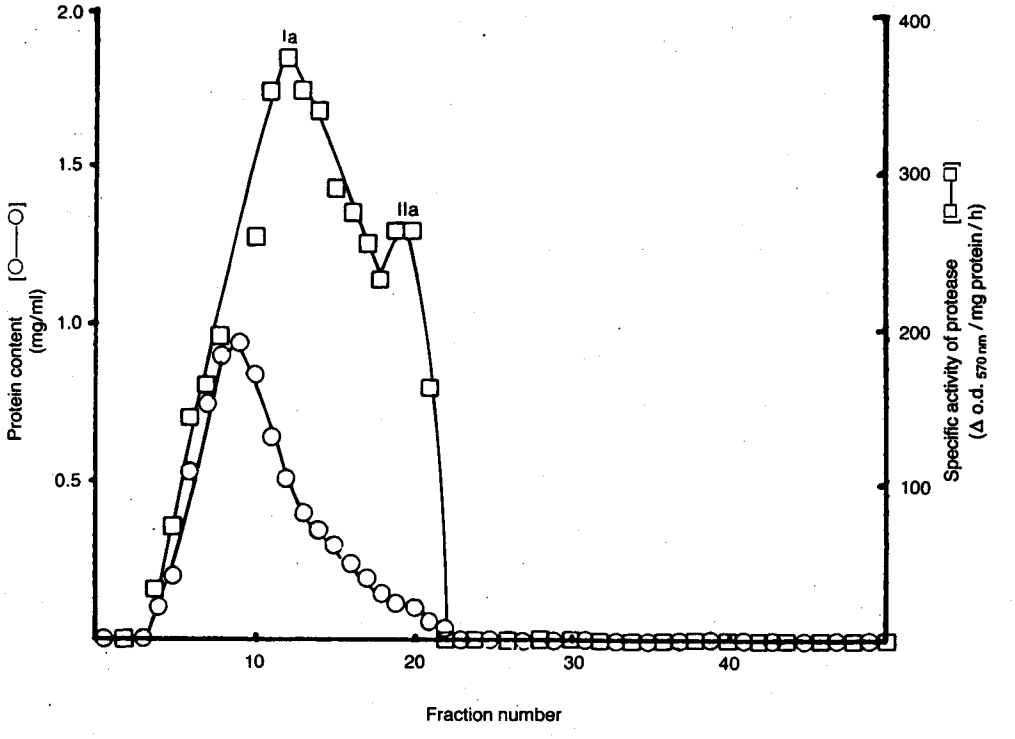
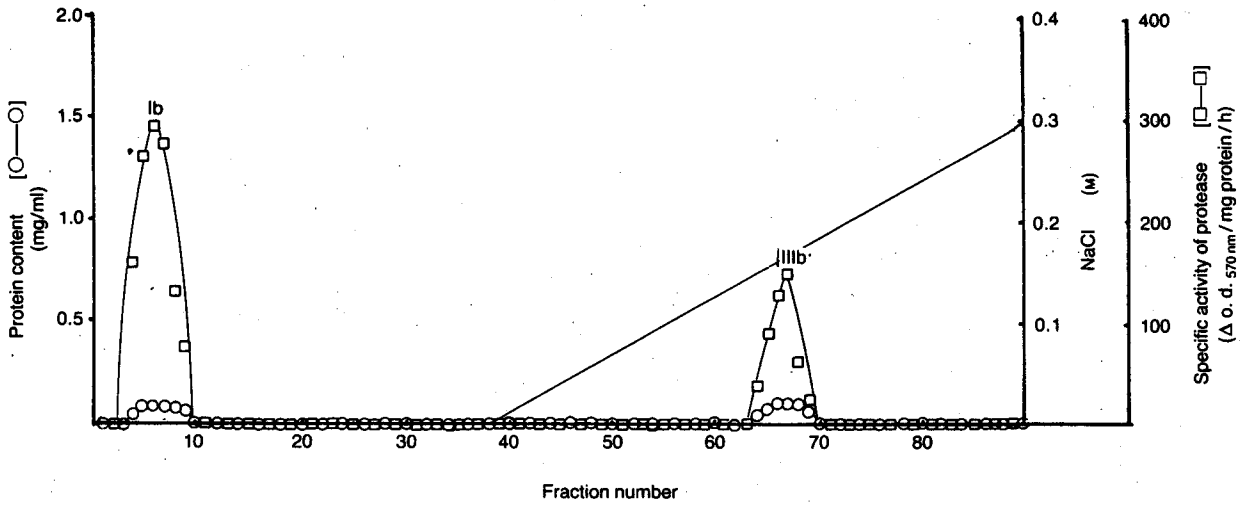


Figure 7.
SP-Sephadex chromatography of the I_a fraction from Sephacryl S-200 chromatography in tobacco calli.



DISCUSSION

The present results on pH profiles and responses to selected chemicals of proteases suggest the predominance of acidic sulfhydryl protease in tobacco leaf and callus. In the leaf of many plant species, sulfhydryl protease has been found to have similar characteristics in acidic pH and in response to the activators 2-mercaptoethanol and ethylenediamine tetraacetic acid and the inhibitor iodoacetic acid (4, 6, 12). In additon, they are capable of digesting animal proteins, hemoglobin and casein, and Fraction I protein (3, 17). These characteristics also held true for the sulfhydryl protease in tobacco. Tobacco leaf protease digested casein inefficiently, which

Table 2. Effect of chemical inhibitors on partially purified proteases from tobacco leaf.*

Chemical inhibition	Protease fraction					
	Peak I_b		Peak II_b		Peak III_b	
	specific activity	percent inhibition	specific activity	percent inhibition	specific activity	percent inhibition
Control	133.4 ± 5.2	—	83.8 ± 10.8	—	142.7 ± 10.6	—
Ethylenediamine tetraacetic acid	203.5 ± 33.0	+ 52%	134.9 ± 15.7	+ 61%	171.2 ± 14.8	+ 20%
Phenylmethane sulfonyl fluoride	86.7 ± 16.3	− 35%	41.9 ± 6.4	− 50%	56.9 ± 11.8	− 60%
Iodoacetic acid	0	−100%	0	−100%	0	−100%
Pepstatin A	98.7 ± 8.2	− 25%	60.5 ± 16.6	− 38%	80.4 ± 9.8	− 44%

* Specific activity is expressed in terms of Δ o.d.$_{570\,nm}$ / mg protein / h (o.d. = optical density).
Data are mean ± S. E. on the basis of eight determinations in two experiments.

substantiates the findings with other plant proteases (4, 6). Casein could be digested effectively at pH 6.5 by proteases of mature corn leaf (5). Proteases from mature tobacco leaf in the present study also exhibited a higher activity in the digestion of casein at neutral pH (Figure 2).

Tobacco leaf and callus represent different morphological and physiological states of plant cells. This appears to exert effects on protease activity. Higher protease activities were obtained from callus than from leaf with either hemoglobin or Fraction I protein as the substrate. Variation of protease activity with plant parts has been reported in potato (14). The present results showed tissue difference not only in specific activity of proteases but also in the composition of molecular variants. In tobacco leaf, three variants were resolved by gel permeation, whereas only two were observed in callus. Cation-exchange column chromatography of the major protease fraction resolved three protease peaks for leaf tissue and two peaks for callus tissue. Since the autolysis of proteases is a well-recognized phenomenon (4), one may suspect the low molecular weight variants as being autolytic products. However, this does not explain why the number of autolytic products differs between leaf and callus proteases. It is therefore concluded that there are at least five sulfhydryl protease variants in tobacco leaf and three in tobacco callus. Whether these variants represent isozymes needs further investigation.

Fraction I protein accounts for more than one-half the soluble protein in fully expanded green leaf and catalyzes CO_2 fixation during photosynthesis (7). Tobacco proteases digested hemoglobin and Fraction I protein optimally at pH 5, but the latter at a lower rate. Similar results were observed with wheat leaf protease using Fraction I protein as the substrate (12). These results are consistent with our recent finding that Fraction I protein can be utilized for growth by tobacco suspension cells in the Murashige-Skoog medium devoid of inorganic nitrogen compounds (8). It is clear that proteases capable of digesting Fraction I protein exist not only in normal tobacco tissue (leaf) but also in abnormal tissue (callus). Tobacco cell culture could therefore provide a useful system in the investigation of genetic and epigenetic regulation of proteases and protein metabolism in higher plants.

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Acknowledgements

This research was supported in part by the U. S. Department of Agriculture Cooperative Agreement No. 58—7B30—0—88 and in part by the University of Kentucky Tobacco and Health Research Institute Project No. 24110. This paper is published with the approval of the Director of the Kentucky Agricultural Experiment Station as Journal Series Paper No. (82—11—195).

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