

Degradation of the Major Storage Protein of *Phaseolus vulgaris* during Germination¹

ROLE OF ENDOGENOUS PROTEASES AND PROTEASE INHIBITORS

Received for publication June 27, 1983 and in revised form October 28, 1983

S. SUZANNE NIELSEN² AND IRVIN E. LIENER*

Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55108

ABSTRACT

Cotyledons from *Phaseolus vulgaris* L. (var. Improved Tendergreen) were tested for their activity on α -N-benzoyl-DL-arginine-p-nitroanilide (BAPNA) and azocasein during a germination period of 10 days. Both activities increased throughout germination when activity was expressed on the basis of dry weight or protein. That these two activities were most likely due to the action of different enzymes was indicated by the fact that (a) optimal pH for the hydrolysis of BAPNA and azocasein was 8.2 and 5.5, respectively, and (b) the digestion of azocasein was considerably enhanced by mercaptoethanol and partially inhibited by thiol protease inhibitors, N-ethylmaleimide, and E-64, whereas these same reagents caused little change in activity toward BAPNA. The three subunits of the major storage protein, G1, disappeared during germination and were accompanied by the accumulation of lower molecular weight products. The breakdown of G1 by extracts of the germinated beans could be demonstrated *in vitro* at pH 5 to 6. This activity was enhanced by mercaptoethanol and completely abolished by N-ethylmaleimide, leupeptin, and E-64. It is concluded that a thiol protease with an acid pH optimum is primarily responsible for the disappearance of the major storage protein during germination. Although an inhibitor of the plant thiol protease, papain, is present in the mature bean and decreases during germination, its role in the control of the breakdown of the storage protein remains to be elucidated.

Legumes constitute a valuable source of protein for large segments of the world's population, but this protein is of poor nutritional value unless subjected to heat treatment (28). Although the beneficial effect of heat treatment has been generally attributed to the destruction of protease inhibitors and lectins (20), other lines of evidence (26, 27) would suggest that the resistance of the native storage protein of *Phaseolus vulgaris* to proteolytic attack by mammalian digestive enzymes may be an important factor contributing to the poor nutritive value of the unheated bean. Little is known, however, as to why the major storage protein of *P. vulgaris* manifests this resistance to proteolytic degradation. Since extensive breakdown of the storage protein is known to occur during germination (9, 30) a study of the manner in which this occurs provides a contrasting situation that might help to explain the restricted digestion of reserve storage protein by mammalian digestive enzymes. In this paper,

we report the results of a study of the breakdown of the major storage protein of *P. vulgaris* in relation to concomitant changes in the protease and protease inhibitor activities that occur during germination of the cotyledons.

EXPERIMENTAL

Germination Studies. Seeds of the French bean, *Phaseolus vulgaris* L. var Improved Tendergreen (purchased from Sun Seeds, Bloomington, MN), were soaked for 5 min in a solution of 0.5% NaOCl followed by rinsing (5 times) with distilled H₂O. The seeds were rolled between wetted sheets of standard germination paper (Anchor Paper Co., St. Paul, MN). The rolls of seeds were kept in plastic bags in a dark room maintained by 25°C and watered periodically to ensure saturation with moisture. Seeds were harvested daily for 10 d, during which time the cotyledons were carefully excised from all other portions of the seed. A known number of cotyledons were homogenized in distilled H₂O in a Polytron tissue homogenizer and lyophilized.

Enzyme Assays. Lyophilized samples of the cotyledons were prepared for enzyme assays by extraction with 0.1 M Tris buffer containing 0.02 M CaCl₂, pH 8.2 (10 mg/ml buffer) followed by centrifugation at 8000g for 3 min. Aliquots of the supernatant were assayed for TLA³ using BAPNA as the substrate (18) and for ACA by the method of Charney and Tomarelli (8). One unit of activity was expressed as an increase of 0.01 absorbance units/min at 410 nm for TLA and at 366 nm for ACA under the conditions defined for each of these assays. Experiments were also conducted in which these activities were measured in the presence and absence of various concentrations of mercaptoethanol, N-ethylmaleimide, TLCK, TPCK, PMSF, leupeptin, and E-64. The trypsin and papain inhibitor activities of bean extracts were determined by their ability to inhibit the activity of bovine trypsin (2× crystallized, Worthington) on BAPNA or of papain (2× crystallized, Sigma) on azocasein. Inhibitor units were then expressed in terms of the number of units of the protease which were inhibited in each of these two systems.

Isolation of the Major Storage Protein (G1). The major storage protein of *P. vulgaris*, which comprises 50% to 75% of the total protein of the seed (5), has been variously referred to as phaseolin (22), fraction E (17), glycoprotein II (2), globulin G1 fraction (13), α -component (16), and vicilin (6). The major storage protein used in this study was isolated by the method of Hall *et al.* (13) who referred to their preparation as the G1 fraction, and for

¹ This work was supported by United States Public Health Service Grant AM 18324.

² Present address: Department of Food Science, Purdue University, West Lafayette, IN 47907.

³ Abbreviations: TLA, trypsin-like activity; BAPNA, α -N-benzoyl-DL-arginine-p-nitroanilide; ACA, activity on azocasein; TLCK, tosyl-L-lysine-chloromethylketone; TPCK, tosylamide-2-phenylethyl-chloromethylketone; PMSF, phenylmethylsulfonyl fluoride; E-64, N-(N-[L-3-trans-carboxoxiran-2-carbonyl]-L-leucyl)-agmatine.

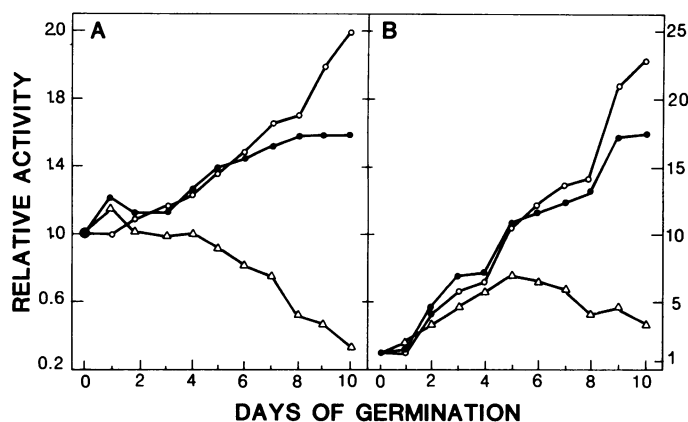


FIG. 1. Changes in TLA (A) and ACA (B) during germination. Relative activities are expressed in relation to activity observed at zero time. ○—○, based on dry weight; ●—●, based on protein content; △—△, per cotyledon.

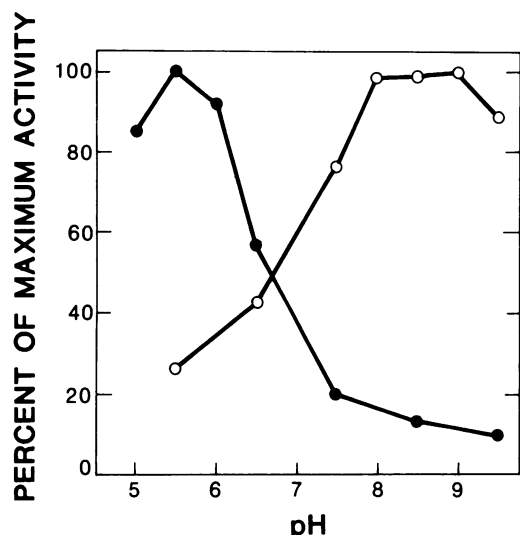


FIG. 2. Effect of pH on the activity of extract of germinated seeds (7 d) toward BAPNA and azocasein. ○—○, activity toward BAPNA (TLA); ●—●, activity toward azocasein (ACA). The following buffers (0.1 M) were used for the indicated pH ranges; citrate, pH 5 to 6.0; imidazole, pH 6.5; Tris, pH 7.5 to 8.5; glycine, pH 9.5.

Table I. Effect of Various Compounds on the Activity of Germinated (6-Day) Cotyledons on Three Different Substrates

Compound Tested	Activity as % of the Control		
	BAPNA ^a	Azocasein ^b	G1 ^c
Control	100	100	100
Mercaptoethanol (10 mM)	102	276	386
N-ethylmaleimide (10 mM)	90	48	0
Leupeptin (10 μM)	44	80	20
E-64 (10 μM)	100	27	0
TLCK (0.5 mM)	17	84	99
TPCK (0.1 mM)	65	94	98
PMSF (2.5 mM)	60	94	98

^a Assayed at pH 8.2 in presence of 0.02 M CaCl₂ (18). Referred to as TLA in text.

^b Assayed at pH 5.5 (8). Referred to as ACA in text.

^c Based on disappearance of the three subunits of G1 separated by SDS-electrophoresis ("Experimental").

that reason we have retained the same designation in this paper.

Gel Electrophoresis. Lyophilized extracts of cotyledons (10 mg) were suspended in 0.5 ml of 0.025 M citrate/phosphate buffer, pH 5.7, containing 0.02% NaN₃ and 10 mM mercaptoethanol. After clarification by centrifugation, 15 μl aliquots were subjected to SDS electrophoresis at pH 8.8 by the method of Laemmli (19) as modified by Ma and Bliss (21) for the separation of the subunits of G1. Electrophoresis was conducted in a Hoefer model E apparatus, and, following staining with Coomassie Blue, the gels were scanned at 600 nm in a Beckman 25 spectrophotometer equipped with a chart recorder. The disappearance of G1 subunits during germination or after exposure of G1 to extracts of germinating beans was calculated from the weight of these peaks which had been carefully cut from the chart.

Protein. The protein content of bean extracts was determined by the method of Hartree (15).

RESULTS AND DISCUSSION

Changes in Protease Activity during Germination. Figure 1 shows the changes in TLA and ACA that take place during germination when these activities are calculated as the ratio of the activity at any given time to the activity at 0 d of germination. Somewhat different patterns are obtained depending on whether these ratios are expressed on the basis of dry weight, protein content, or per cotyledon. On a weight or protein basis, TLA displays a slow but steady increase after 3 to 4 d of germination. When TLA is expressed on a per cotyledon basis, however, a marked drop in activity occurs after 4 d, until only about 30% of the activity prior to germination remains after 10 d. This disparity arises from the fact that the dry weight and protein content of each cotyledon was found to decrease during germination. This would also explain why Pusztai and Duncan (24) found that the activity of *P. vulgaris* toward BAPNA showed little change during germination when expressed on a weight basis, whereas Crump and Murray (10) reported a decrease in this activity on a per cotyledon basis.

The pattern of the changes in ACA during germination was somewhat different from that of TLA. The increases in ACA on a dry weight or protein basis were much more pronounced, and, on a per cotyledon basis, this activity increased and reached a maximum after 5 d of germination whereupon it began to decline steadily. The latter observation agrees with the results of others (12, 30) who have also reported that the activity of *P. vulgaris* extracts on casein or azocasein peaks at 5 or 6 d of germination.

It is important to point out that, in the experiments just described, the activities on the synthetic substrate BAPNA and azocasein were conducted at pH 8.2 and 5.5, respectively. The choice of a different pH for each of these two substrates was dictated by the results of a study of the effect of pH on the activity of 7-d germinated beans (Fig. 2). The optimum pH for activity on BAPNA was in the range of 8.2 to 9, whereas maximum activity on azocasein occurred at pH 5.5. There was, therefore, a possibility that the changes in TLA and ACA activities recorded in Figure 1 might be due to two different kinds of proteases.

More definitive evidence that TLA and ACA activities were in fact the manifestation of dissimilar enzymes came from studies involving the effects of mercaptoethanol and various inhibitors (Table I). Mercaptoethanol produced almost a 3-fold enhancement of ACA but had no effect on TLA. This result, coupled with the fact that ACA was partially inhibited by N-ethylmaleimide, would indicate that the enzyme responsible for a portion of the azocaseinolytic activity of the germinated bean is a sulfhydryl enzyme. E-64, which has been shown to be an effective inhibitor of thiol proteases (14), also inhibited ACA to a significant extent but had no effect on TLA. Leupeptin, a known inhibitor of serine and thiol proteases (29), also proved to be an effective

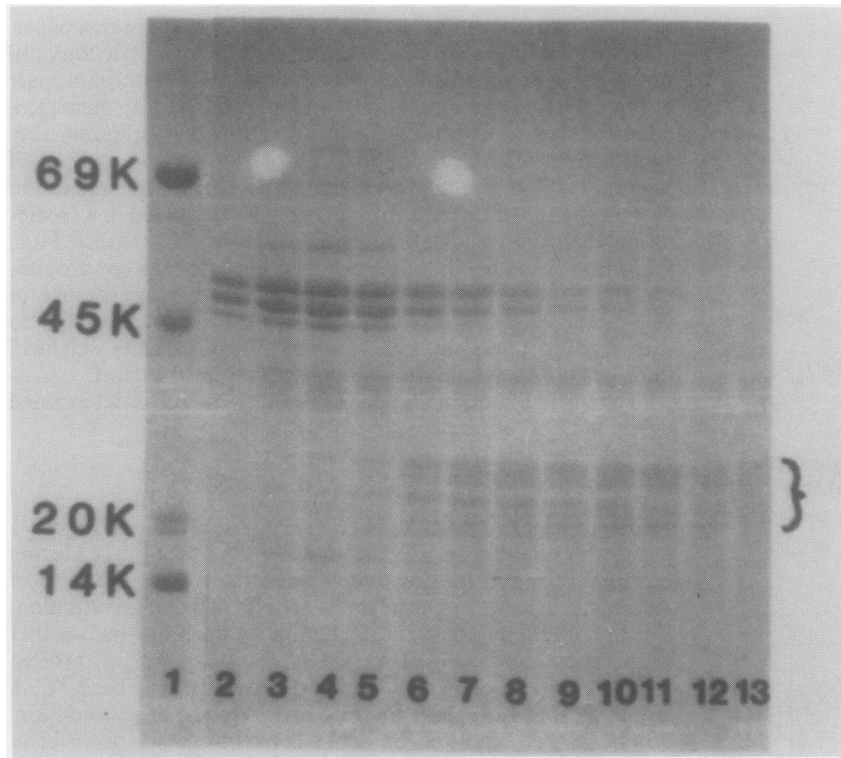


FIG. 3. SDS electrophoresis patterns of germinating cytoledons. Lane 1, mol wt markers consisting of BSA (69K), ovalbumin (45K), Kunitz soybean trypsin inhibitor (20K), and lysozyme (14K); lane 2, purified G1 fraction; lane 3, mature bean prior to germination (zero time); lanes 4 to 13, after 1 to 10 d of germination, respectively. Brackets denote protein bands which appear to represent the degradation products of G1.

inhibitor of TLA, but somewhat less effective against ACA. The well known inhibitors of serine proteinases, TLCK, TPCK, and PMSF, also inhibited TLA but were relatively ineffective against ACA.

The general conclusion that could be drawn from the studies thus far described is that the germination of *P. vulgaris* is accompanied by an increased production of at least two different proteases, one of which is an -SH protease most active at pH 5.5 and the other a protease which resembles trypsin based on its ability to hydrolyze BAPNA under alkaline conditions.

A comparison of the effect of mercaptoethanol and various inhibitors on the ability of a germinated bean extract (6 d) to digest azocasein and G1 is included in Table I. Mercaptoethanol proved to be a very potent activator of the enzyme responsible for the degradation of G1. The serine proteinase inhibitors, PMSF, TLCK, and TPCK, failed to inhibit the degradation of G1, whereas the SH enzyme inhibitors *N*-ethylmaleimide, leupeptin, and E-64 (14) completely abolished this activity. It will be noted, however, that these last three inhibitors only partially inhibited the digestion of azocasein. This indicates that the hydrolysis of azocasein can only be partially accounted for by an SH protease so that the presence of non-SH proteases active at pH 5.7 cannot be discounted. It is evident, however, that the enzyme responsible for the breakdown of G1 must be an SH protease. In this connection, Alpi and Beevers (1) have shown that leupeptin strongly inhibited their germination of castor beans which they also attributed to the inhibition of an SH protease involved in the mobilization of storage protein.

Breakdown of G1 during Germination. SDS-electrophoresis patterns of extracts of cotyledons which had germinated for various periods of time are shown in Figure 3. The purified G1 fraction, shown in lane 2, was comprised of three subunits with mol wt of 51,000, 48,000, and 45,000. These correspond to the same three subunits reported by Brown *et al.* (7) for the Improved Tendergreen variety of *P. vulgaris*. As germination proceeded there was a gradual disappearance of these subunits and a concomitant increase in components having mol wt in the range of

20,000 to 30,000. The rate of the breakdown of each of these subunits could be quantitated by the decrease in the areas of each of these peaks (as measured by the weights of the excised peaks from the recording charts). Figure 4 shows some typical scans of the gels obtained after 0, 3, 6, and 9 d of germination, and Figure 5 shows the rates at which each of the three subunits disappear. The smallest of these subunits with a mol wt of 45,000 was degraded more rapidly than the two larger subunits which were degraded somewhat more slowly but at parallel rates. This pattern of storage protein breakdown differs from that reported for the mung bean (5) and the pea (3) where the largest subunit of the major storage protein of these plants was the first to be broken down during germination.

Although the increase in proteolytic activities (expressed on a dry weight or protein basis) during germination previously noted would appear to be related to the disappearance of G1, more direct evidence for the association of these two phenomena came from experiments in which extracts of the germinated beans were added to G1 as the substrate. Preliminary studies indicated that maximum degradation of G1 by germinated bean extracts occurred in the pH range of 5 to 6, indicating that the thiol protease was most likely responsible for this effect. Extracts from beans which had germinated from 1 to 10 d were incubated with G1 at pH 5.7, and the gels which were obtained were scanned for measuring the amount of G1 that remained in each system (Fig. 6). The pattern of the breakdown of the G1 subunits shown here was remarkably similar to that observed in extracts of the bean itself (Fig. 5).

Changes in Proteinase Inhibitor Activity during Germination. Since an SH protease appeared to be the major factor controlling the breakdown of the major storage protein of *P. vulgaris* during germination, evidence was sought for the presence of an inhibitor of this enzyme which might serve to modulate the activity of this enzyme during germination. The presence of an inhibitor of the SH protease papain in dry seeds of *P. vulgaris* has been reported (25), but no information is available on changes that might ensue during germination. Figure 7 shows that the level of papain

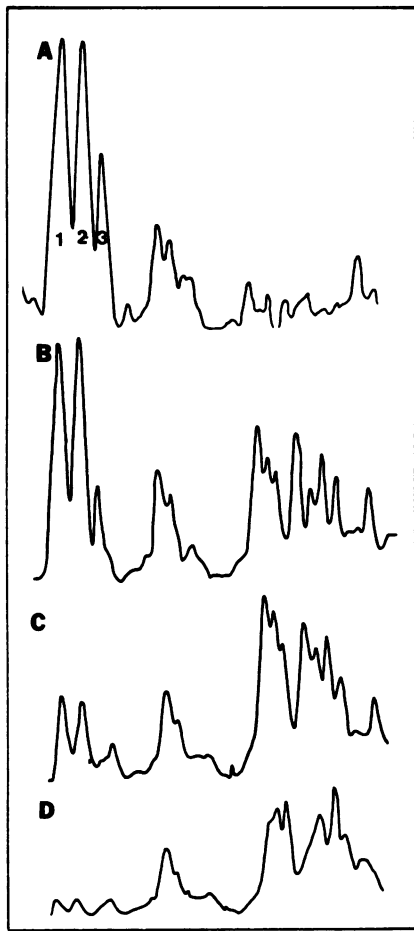


FIG. 4. Scans of gel patterns of germinating cotyledons. A, mature bean prior to germination (zero time); B, after 3 d; C, after 6 d; D, after 9 d. The three peaks observed in the ungerminated sample (A) are those of the three subunits of G1 where 1 = 51K, 2 = 48K, and 3 = 45.5K.

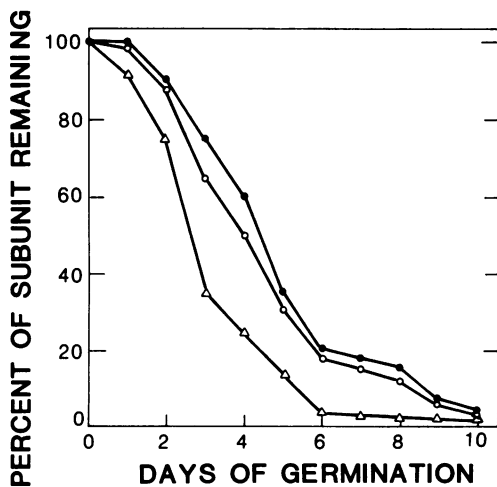


FIG. 5. Changes in subunit composition of G1 during germination. Amounts of each subunit during the course of germination were based on the weight of each of the peaks corresponding to the three subunits (see Fig. 4) cut from the recording paper of a gel scan. These values were then used to calculate percentage of each subunit remaining. ○—○, subunit 1 (51K); ●—●, subunit 2 (48K); △—△, subunit 3 (45.5K).

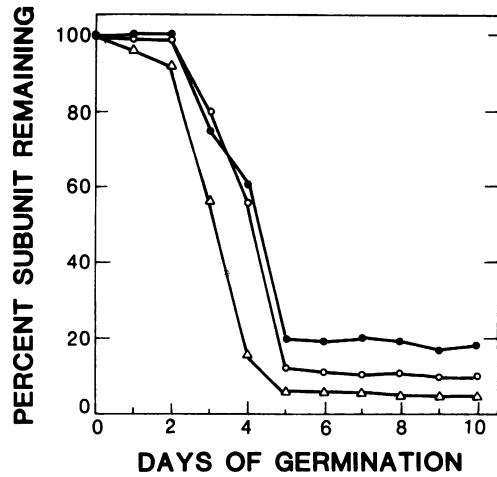


FIG. 6. Breakdown of G1 by extracts of cotyledons which had germinated from 1 to 10 d. A suspension of 50 mg lyophilized cotyledons per ml 0.025 M citrate/phosphate buffer, pH 5.7, containing 10 mM mercaptoethanol, was centrifuged and clarified by filtration through glass wool. To 0.4 ml of this extract was added 0.6 mg G1 and the mixture was incubated at 37°C for 15 h. SDS-electrophoresis was performed ("Experimental"), and the breakdown of G1 subunits was quantitated as described for Figure 5. Corrections were made for any G1 subunits present in controls containing the bean extracts without added G1. ●—●, 51K subunit; ○—○, 48K subunit; △—△, 45.5 K subunit.

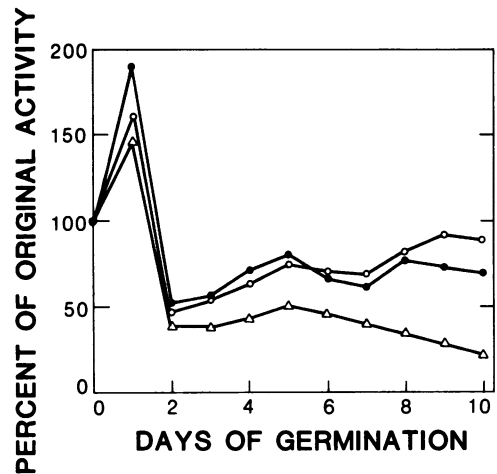


FIG. 7. Papain inhibitor activity during germination. Extracts of germinated cotyledons were mixed with 10 μg papain in a final volume of 1ml of 0.1 M phosphate buffer, pH 7.5, containing 0.02 M cysteine and allowed to incubate for 10 min at 37°C. Residual activity on azocasein was determined at pH 7.5 as described in "Experimental." ○—○, based on dry weight; ●—●, based on protein content; △—△, per cotyledon.

inhibitor activity rose dramatically after the first day of germination and then rapidly declined to about 20% (on a per cotyledon basis) or 70% to 80% (on a dry weight or protein basis) of the original activity in the dry seed. These low levels of activity persisted throughout the remainder of the 10-d germination period. Since this decrease in papain inhibitor activity coincided with the increase in SH protease activity as measured on azocasein (Fig. 1B) and the disappearance of G1 (Figs. 4 and 5), it is tempting to speculate that the degradation of the major storage protein may be attributed to an SH protease which is inhibited in the dry seed but becomes active during germination when the level of its inhibitor becomes depressed. Thus far, however, proof is lacking that the SH protease is in fact inhibited by the same factor which inhibits papain. Attempts to purify the papain

inhibitor are currently in progress and, if successful, will permit the experimental verification of this hypothesis. Baumgartner and Chrispeels (4, 5) have shown that, although the mung bean possesses an inhibitor of an endopeptidase responsible for degrading vicilin, the major storage protein of this seed, their kinetic data did not support the concept that this inhibitor serves to regulate the increase in the activity of this enzyme during germination. It may be that the increase in the activity of the thiol proteinase in our studies may simply represent an enhanced *de novo* synthesis of this enzyme as suggested by Yomo and Srinivasan (30).

Unlike the decrease in papain inhibitor activity which occurred after the first day of germination, no significant change in trypsin inhibitor activity could be discerned up to 8 d of germination (data not shown). Other investigators have also reported little (23) or no (11) change in trypsin inhibitor activity to occur during the germination of *P. vulgaris*. Moreover, the finding that synthetic inhibitors of trypsin such as PMSF and TLCK failed to prevent the degradation of G1 by extracts of the germinated bean (Table I) would indicate that endogenous trypsin inhibitor probably does not play any role in the control of the mobilization of the reserve protein of this legume during germination.

LITERATURE CITED

- ALPI A, H BEEVERS 1981 Effects of leupeptin on proteinase and germination of castor beans. *Plant Physiol* 68: 851-853
- BARKER RD, E DERBYSHIRE, A YARWOOD, D BOULTER 1976 Purification and characterization of the major storage proteins of *Phaseolus vulgaris* seeds, and their intracellular and cotyledonary distribution. *Phytochemistry* 15: 751-757
- BASHA SMM, L BEEVERS 1975 The development of proteolytic activity and protein degradation during the germination of *Pisum sativum* L. *Planta (Berl)* 124: 77-87
- BAUMGARTNER B, MJ CHRISPEELS 1976 Partial characterization of a protease inhibitor which inhibits the major endopeptidase present in cotyledons of mung beans. *Plant Physiol* 58: 1-6
- BAUMGARTNER B, MJ CHRISPEELS 1977 Purification and characterization of vicilin peptidohydrolase, the major endopeptidase in the cotyledons of mung-bean seedlings. *Eur J Biochem* 77: 223-233
- BOULTER D, IM EVANS, E DERBYSHIRE 1973 Proteins of some legumes with reference to environmental factors and nutritional value. *Qual Plant Plant Foods Hum Nutr* 23: 239-250
- BROWN JWS, Y MA, FA BLISS, TC HALL 1981 Genetic variation in the subunits of globulin-1 storage protein of French bean. *Theor Appl Genet* 59: 83-88
- CHARNEY J, RM TOMARELLI 1947 A colorimetric method for the determination of the proteolytic activity of duodenal juice. *J Biol Chem* 171: 501-505
- CHRISPEELS MJ, D BOULTER 1975 Control of storage protein metabolism in the cotyledons of germinating mung beans: role of endopeptidase. *Plant Physiol* 55: 1031-1037
- CRUMP JA, DR MURRAY 1979 Proteolysis in cotyledon cells of *Phaseolus vulgaris* L.: Changes in multiple hydrolase activities following germination. *Aust J Plant Physiol* 6: 467-474
- EL-HAG N, NF HAARD, RE MORSE 1978 Influence of sprouting on the digestibility coefficient, trypsin inhibitor and globulin proteins of red kidney beans. *J Food Sci* 43: 1874-1875
- FELLER U 1979 Nitrogen mobilization and proteolytic activities in germinating and maturing bush beans (*Phaseolus vulgaris* L.). *Z Pflanzenphysiol* 95S: 413-422
- HALL TC, RC MCLEESTER, FJA BLISS 1977 Equal expression of the maternal and paternal alleles for the polypeptide subunits of the major storage protein of the bean *Phaseolus vulgaris* L. *Plant Physiol* 59: 529-536
- HANADA K, M TAMAI, S OHMURA, J SAUVADA, T SEKI, I TANAKA 1978 Structure and synthesis of E-64, a new thiol proteinase inhibitor. *Agric Biol Chem* 42: 529-536
- HARTREE EF 1972 Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal Biochem* 48: 422-427
- ISHINO K, DML ORTEGA 1975 Fractionation and characterization of major reserve proteins from seeds of *Phaseolus vulgaris*. *J Agric Food Chem* 23: 529-533
- JAFFE WG, K HANNIG 1965 Fractionation of proteins from kidney beans (*Phaseolus vulgaris*). *Arch Biochem Biophys* 109: 80-91
- KAKADE ML, N SIMONS, IE LIENER 1969 Evaluation of natural versus synthetic substrates for measuring the antitryptic activity of soybean samples. *Cereal Chem* 46: 518-526
- LAEMMLI UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685
- LIENER IE 1978 Protease inhibitors and other toxic factors in seeds. In G Norton, ed, *Plant Proteins*. Butterworths, London, pp 117-140
- MA Y, FA BLISS 1978 Seed proteins in common bean. *Crop Sci* 18: 431-437
- OSBORNE TB 1894 The proteins of the kidney bean. *J Am Chem Soc* 16: 633-643
- PALMER R, A MCINTOSH, A PUSZTAI 1973 The nutritional evaluation of kidney beans (*Phaseolus vulgaris*). The effect on nutritional value of seed germination and changes in trypsin inhibitor content. *J Sci Food Agric* 24: 937-944
- PUSZTAI A, I DUNCAN 1971 Changes in proteolytic enzyme activities and transformation of nitrogenous compounds in the germinating seeds of kidney bean (*Phaseolus vulgaris*). *Planta (Berl)* 96: 317-325
- RELE MV, HG BARTAK, V JAGANNATHAN 1980 Proteinase inhibitors from *Vigna unguiculata* subsp. *cylindrica*. I. Occurrence of thiol proteinase inhibitors in plants and purification from *Vigna unguiculata* subsp. *cylindrica*. *Arch Biochem Biophys* 304: 117-128
- ROMERO J, DS RYAN 1978 Susceptibility of the major storage protein of the bean, *Phaseolus vulgaris* L., to *in vitro* enzymatic hydrolysis. *J Agric Food Chem* 26: 784-788
- THOMPSON RM, IE LIENER 1980 *In vitro* and *In vivo* studies on the digestibility of the major storage protein of the navy bean (*Phaseolus vulgaris*). *Qual Plant Plant Foods Hum Nutr* 30: 13-25
- TOBIN JG, KJ CARPENTER 1978 The nutritional value of the dry bean (*Phaseolus vulgaris*). A literature review. *Nutr Abstr Rev Ser A* 48: 919-936
- UMEZAWA H 1982 Low molecular weight enzyme inhibitors of microbial origin. *Annu Rev Microbiol* 36: 75-99
- YOMO H, K SRINIVASAN 1973 Protein breakdown and formation of protease in attached and detached cotyledons of *Phaseolus vulgaris* L. *Plant Physiol* 52: 671-673