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PROTEOLYTIC AND TRYPSIN INHIBITORY ACTIVITIES IN EXTRACTS OF GERMINATING *PISUM SATIVUM* SEEDS

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Key Word Index—*Pisum sativum*; Leguminosae; pea; germinating seed; trypsin inhibitor; proteolysis.

Abstract—A rise in azoglobulytic activity and a fall in trypsin-inhibitory activity has been detected in extracts of germinating pea seeds free from microbial infection. By investigating the subcellular distribution of protease activity, trypsin-inhibitory activity and the effect of the inhibitor on endogenous protease activity, no convincing evidence could be obtained to suggest that the trypsin-inhibitory activity was involved in the regulation of proteases during germination.

INTRODUCTION

A NUMBER of conflicting observations have been made on the changes in proteolytic activity present in extracts of germinating pea seeds.[1–4] Much of this work can be criticized on two grounds: (a) the use of assays of proteolytic activity which are based on the measurement of increase in TCA-soluble material absorbing at 280 nm, and (b) the possibility that some of the activity measured was contributed by micro-organisms growing on the germinating seeds.

In the work described here we have tried to overcome these two criticisms by using a reliable assay for measuring proteolytic activity and also extracting pea seeds which were free from infection by micro-organisms. For comparative purposes we have also followed changes in L-leucine-*p*-nitroanilidase (LPA-ase) and α-benzoyl-*p*-nitroanilidase (BAPA-ase) activities present in the extracts, since it is known that these two activities fall rapidly during germination[4] and there is some evidence to suggest that the proteins responsible for these activities are distinct from the protein(s) responsible for proteolytic activity.[4]

In the majority of instances where increases of proteolytic activity have been detected, the mechanism responsible for the increase has not been investigated. It is often suggested that *de novo* enzyme synthesis or zymogen activation is responsible. Other mechanisms which might lead to increases in activity are seldom considered. Because legume seeds are rich sources of trypsin-inhibitory activity, we have attempted to assess the role played by this activity in the regulation of proteolysis during the germination of pea seeds.

RESULTS AND DISCUSSION

Figure 1 shows that azoglobulytic activity at pH 7·0 of extracts prepared from sterile pea seed cotyledons as germination proceeds. This observation agrees with some of the

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[1] YOUNG, J. L. and VARNER, J. E. (1959) *Arch. Biochem. Biophys.* **84**, 71.
[2] HENSHALL, J. D. and GOODWIN, T. W. (1964) *Phytochemistry* **3**, 677.
[3] MORRIS, J. (1968) Ph.D. Thesis, University of Liverpool.
[4] BEEVERS, L. (1968) *Phytochemistry* **7**, 1837.

earlier observation on peas, though Young and Varner,[1] and Henshall and Goodwin,[2] using casein as substrate and the Kunitz assay, could not detect any significant increases. However, it has been shown[5] that these workers used an assay in which any changes in protease activity may have been masked by the activity of ribonuclease.

We have found the azoglobulin assay highly suitable for the assay of proteases in crude extracts of seeds, though the adsorption of azopeptides by TCA-precipitated protein during the assay requires comment. Azopeptides can be washed off proteins precipitated by TCA, the quantity of azopeptide adsorbed depending upon the concentration of protein in the assay solutions. In most cases where the concentration of protein remains constant these effects are not important. However, when the protein concentration varies, the quantity of azopeptide adsorbed will also vary and so affect the assay; presumably this criticism applies to any method of estimating proteolysis which involves precipitating the substrate with TCA.

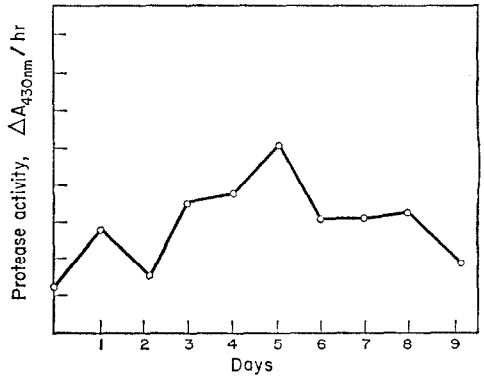

FIG. 1. CHANGES IN THE PROTEOLYTIC ACTIVITY OF PEA SEED COTYLEDON EXTRACTS DURING GERMINATION, MEASURED BY THE AZOGLOBULIN ASSAY.

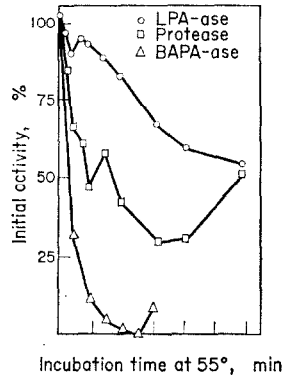

FIG. 2. THERMAL DENATURATION CURVES AT 55° OF AZOGLOBULYTIC ACTIVITY, BAPA-ASE AND LPA-ASE ACTIVITIES PRESENT IN EXTRACTS OF PEA SEED COTYLDEONS.

Of the ten seeds taken each day for sterility checks, only one seed per sample was found to be infected on days 4, 7, 8 and 9, and two seeds on day 6. These are likely to be overestimates as the tests were carried out on full nutrient agar, while the seeds used for enzyme assays were grown on damp tissues. We therefore feel that the proteolytic activity which we have measured is of seed and not microbial origin.

Assay of extracts for BAPA-ase and LPA-ase activities, enzymes also possibly involved in some stage of proteolysis, did not show the rise as detected for azoglobulytic activity, but declined in activity as described by Beevers.[4] This suggests that the protein(s) responsible for the azoglobulytic activity are distinct from those responsible for BAPA-ase and LPA-ase activities. This was confirmed by a determination of their denaturation curves (Fig. 2).

The high levels of trypsin-inhibitory activity detected in ungerminated seeds, declined as germination proceeded (Fig. 3). Workers[6] failing to detect such activities in pea seeds have

[5] MARRINK, J. and GRUBER, M. (1966) *Biochem. Biophys. Acta* **118**, 438.
[6] BORCHERS, R. and ACKERSON, C. W. (1947) *Arch. Biochem. Biophys.* **13**, 291.

probably used inadequate methods of extraction. Since an increase protein concentration can influence the results of the azoglobulytic and BAPA-ase assays, it is possible that the inhibitory activities present in seed extracts might merely be due to the addition of extra protein to the assay solutions. The pea seed extracts contained decreasing amounts of protein as germination proceeded and would therefore adsorb less peptide material; this would give an apparent relief of trypsin-inhibitory activity. That this was not so was shown by measuring the specific inhibitory activity of a pea seed extract and a 0·1 % casein solution towards the BAPA-ase activity of trypsin (Table 1).

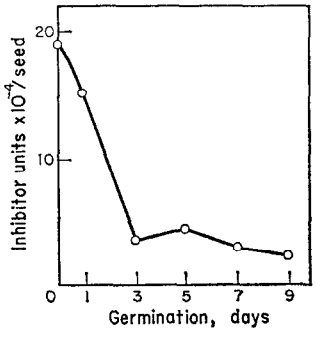

FIG. 3. ILLUSTRATES THE CHANGE IN TRYPSIN-INHIBITOR CONTENT OF PEA SEEDS DURING GERMINATION. INHIBITORY ACTIVITY WAS ASSAYED USING THE AZOGLOBULIN ASSAY OF TRYPSIN.

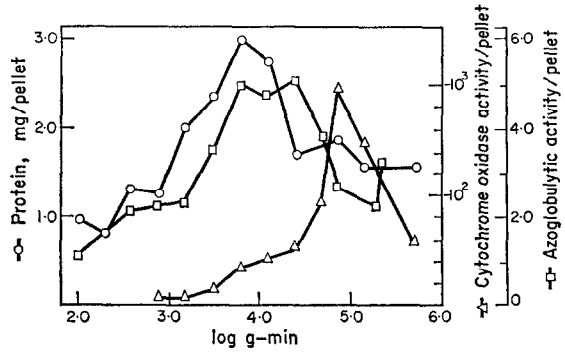

FIG. 4. ILLUSTRATION OF THE SEDIMENTATION OF PROTEIN, AZOGLOBULYTIC ACTIVITY AND CYTOCHROME OXIDASE ACTIVITY WHEN A HOMOGENATE OF PEA SEED COTYLEDONS WAS SUBJECTED TO DIFFERENTIAL CENTRIFUGATION.

The marked decline in inhibitory activity as germination proceeds is at variance with other observations[7] with peas. Possibly these workers failed to recognize that a linear relationship exists between inhibitor concentration and inhibitory activity only up to about 30 % inhibition of trypsin activity and therefore they could have been dealing with saturating amounts of inhibitor both before and after germination.

TABLE 1. THE INHIBITORY ACTIVITIES AND SPECIFIC INHIBITORY ACTIVITIES, ASSAYED WITH TRYPSIN, OF AN EXTRACT OF UNGERMINATED PEA SEEDS AND A 0·1 % CASEIN SOLUTION

	Percentage inhibition of trypsin BAPA-ase activity	Units of inhibitor per ml	Protein (mg/ml)	Specific inhibitory activity (units/mg protein)
Pea extract diluted ×100	33·1	38·0	0·25	149·0
Casein solution 0·1 %	17·4	19·9	1·00	19·9

The decline in inhibitory activity observed here, together with the rise in azoglobulytic activity prompted us to examine the possible role played by the inhibitor in regulating

[7] CHATTOPADHYAY, H. and BANERJEE, S. (1953) *Indian J. Med. Res.* **41**, 185.

proteolysis in these seeds. If the trypsin inhibitor is involved in this function, it would be reasonable to suppose that it might be located at those sites in the cotyledon cells where the protease activity is also located. There is some evidence[8–11] to suggest that certain seed proteases are located in protein bodies, the predominant organelle found in these cells. Therefore protein body fractions were examined for protease and trypsin-inhibitory activity.

Various sub-cellular fractions were prepared from pea seeds as described by Morris[12] and the distribution of azoglobulytic activity amongst these fractions was determined. Of the activity detected in the initial homogenate, 25% was detected in the starch grain fraction, 29% in the protein body fraction, 24% in the mitochondrial fraction, and 7% in the remaining supernatant. In view of this diffuse distribution the sedimentation behaviour of azoglobulytic activity, protein bodies and mitochondria were examined in more detail by differential centrifugation. The sedimentation profile of azoglobulytic activity (pH 5·5) and protein bodies were similar but clearly different from that of cytochrome oxidase activity (Fig. 4). Further, the protein body fraction retained 50% of its protease activity after centrifugation through a discontinuous sucrose gradient. These results suggest that protease activity is associated with the protein body fraction as prepared here. However, they do not exclude the possibility that some or all of the activity is adsorbed on to the particles during their isolation, or that it is associated with different types of particles sedimenting with the protein bodies.

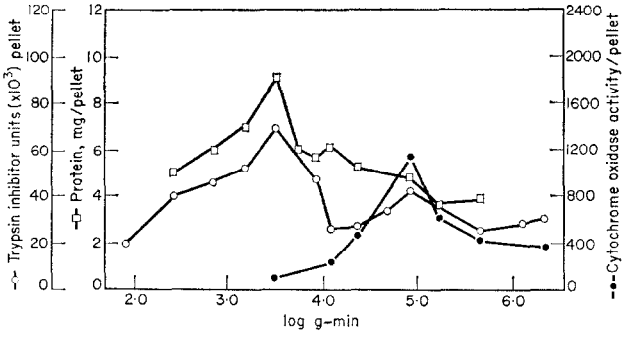

FIG. 5. ILLUSTRATION OF THE SEDIMENTATION OF PROTEIN, TRYPSIN-INHIBITORY ACTIVITY (ASSAYED ON THE BAPA-ASE ACTIVITY OF TRYPSIN) AND CYTOCHROME OXIDASE ACTIVITY, WHEN A HOMOGENATE OF PEA SEED COTYLEDONS WAS SUBJECTED TO DIFFERENTIAL CENTRIFUGATION.

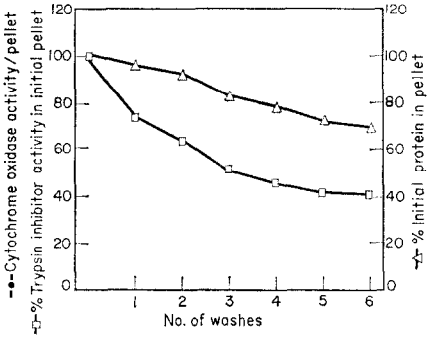

FIG. 6. THE EFFECT OF WASHING ON THE TRYPSIN-INHIBITORY ACTIVITY AND PROTEIN CONTENT OF A PROTEIN BODY FRACTION PREPARED FROM PEA SEED COTYLEDONS.

When a homogenate of pea seeds was separated into protein body and supernatant fractions by centrifugation for 14000 *g*-min[13] it was found that 51% of the trypsin inhibitory activity detected in the initial homogenate was found to be associated with the former fraction and 47% with the latter. Examination of seidmentation profiles shown in Fig. 5 show that a peak of inhibitory activity is associated with the protein body fraction and a further peak of inhibitory activity is correlated with the peak of cytochrome oxidase activity.

[8] MATILE, P. (1968) *Z. Plant. Physiol.* **58,** 365.
[9] YATSU, L. Y. and JACKS, T. J. (1968) *Arch. Biochem. Biophys.* **124,** 466.
[10] ORY, R. L. and HENNINGSEN, K. W. (1969) *Plant Physiol.* **44,** 1488.
[11] ST. ANGELO, A. J., ORY, R. L. and HANSEN, H. J. (1969) *Phytochemistry* **8,** 1135.
[12] MORRIS, G. F. I. (1969) Ph.D. Thesis, University of Liverpool.
[13] MORRIS, G. F. I. THURMAN, D. A. and BOULTER, D. (1970) *Phytochemistry* **9,** 1707.

One interpretation of these data is that the inhibitor is cytoplasmic in origin and that it is adsorbed on to organelle surfaces during extraction. This possibility was investigated in a series of washing experiments. In these experiments it became essential to estimate the damage to protein bodies during washing; this was measured by the release of soluble protein.

Fifty per cent of the inhibitory activity associated with the unwashed protein body fraction could be removed by washing with extractant and then, rebound; unwashed protein body fractions bound negligible amounts of activity. In a further experiment the decline in inhibitory activity and loss of soluble protein from a protein body fraction were followed simultaneously after a number of washes (Fig. 6). The protein contents of the fraction declined linearly, 30% of the initial protein was lost after 6 washes; the decline in inhibitor content followed a different pattern; after 3 washes, 50% of the initial activity was lost, but over the next succeeding 3, only a further 10% was lost. The difference between the steady linear decline of protein content and the more complex loss of inhibitor is crucial. Assuming that the estimate of protein loss to be at least proportional to the damage and leaching of protein bodies, the loss of inhibitory activity would be expected to parallel the loss of protein if both were located inside the protein bodies. The data indicate that this is not the case.

The fact that approx. 50% of the total activity present in homogenates was found in the supernatant fraction, after removal of protein bodies and mitochondria, and of that associated with the bodies over half could be removed by washings, suggests that less than 25% of the total inhibitory activity detected in the initial homogenate is located inside the bodies or tightly bound to them. This argues strongly against a location in the protein bodies, since to obtain such a distribution would require them to be highly unstable in the extractants used; several workers have shown this is not so.[13] Our data suggest that the majority of the trypsin-inhibitory activity present in pea seeds is found outside the protein bodies; this fact would appear to exclude it from regulating proteolysis during germination by inhibition of those proteases actively engaged in proteolysis in these organelles.

TABLE 2. THE EFFECT OF AN EXTRACT OF UNGERMINATED PEA SEEDS ON THE AZOGLOBULYTIC ACTIVITY PRESENT IN EXTRACTS OF PEA COTYLEDONS FROM 5-day-old PEA SEEDLINGS

Extract of ungerminated peas added (ml)	Units of protease activity (ml)	Percentage inhibition of pea protease activity
0·0	0·85	0·0
0·1	0·81	4·7
0·4	0·75	14·1
0·7	0·71	16·5
1·0	0·65	23·5

An alternative approach to studying the possible *in vivo* role of the inhibitor has been to study the effects of the inhibitor upon the azoglobulytic activity *in vitro*. Inhibitor was prepared from ungerminated seeds containing high inhibitor activity. Azoglobulytic activity was extracted from 5-day-old pea seeds possessing a low inhibitor content, inhibition of trypsin activity was detected, but no strong inhibition of azoglobulytic activity was observed (Table 2). The levels of inhibition observed could have been due to the effects of

increased protein concentration on the assay. It would appear unlikely, therefore, that the protease(s) responsible for the breakdown of azoglobulin has its activity regulated during germination by the trypsin inhibitor.

EXPERIMENTAL

Seeds of *Pisum sativum* (garden pea) cv. 'Alaska', were soaked overnight and sterilized in 1% bromine solution for 10 min, rinsed 3× in sterile dist. H_2O and germinated under aseptic conditions in Petri dishes on damp tissues in darkness at 25°. Extracts of the cotyledons for enzymic assay were prepared with 2% KCl in 0·1 M *N*-ethylmorpholine buffer, pH 7·0, by grinding in a mortar at 4°. The homogenate was then centrifuged for 20 min at 25000 *g* and assayed at once. Each batch of seeds extracted was assayed for infection by plating seed samples on to nutrient agar, and incubating at 26°. Azoglobulin was prepared from pea globulin in the manner described by Goad[14] for azoglutelin. The azoglobulin assay was performed by incubating 1 ml of enzyme or seed extract with 1 ml of 1% azoglobulin and 1 ml of 0·1 M *N*-ethylmorpholine, pH 7·0, or 0·1 M citrate, pH 5·5, for various times at 37°. The reaction was terminated by the addition of 1 ml of 20% TCA. The precipitate was centrifuged down at 2500 *g* for 10 min, and 2 ml of the supernatant made alkaline with 2 ml of 2 N NaOH. The change in absorbance at 430 nm of the NaOH-treated supernatant over that of a zero-time mixture, corrected for changes in enzyme and substrate controls, was used as a measure of proteolysis. One unit of tryptic or azoglobulytic activity was defined as that amount of enzyme causing a change in absorbance at 430 m, of 0·001/min, usually at 37°. BAPA-ase and LPA-ase were assayed as described by Erlanger,[15] one unit of each enzyme was defined as that which would cause a change in absorbance at 410 nm of 0·001/min at pH 8·1, and at 28°. Trypsin-inhibitory activity was assayed by measuring its activity towards trypsin using the pH 7·0 azoglobulin assay, described above, or by its activity towards the BAPA-ase activity of tyrpsin. Inhibitor activity was either expressed as a percentage inhibition of control trypsin activity, or in terms of units of inhibitor activity. The unit of inhibitor activity was defined as that amount which would reduce the activity of one trypsin unit (1 azoglobulytic or 1 BAPA-ase unit) to 0·8 trypsin unit. Assays were carried out such that the total inhibition did not exceed 30% of the control trypsin activity. The procedure and extractants used for isolating protein bodies were those described by Morris *et al.*[13] Cytochrome oxidase activity was assayed essentially as described by Smith.[16] Protein was determined by the methods of Lowry *et al.*[17] and Waddel.[18]

[14] GOAD, L. J. (1963) Ph.D. Thesis, University of Manchester.
[15] ERLANGER, B. F., KOKOWSKY, N. and COHEN, W. (1961) *Arch. Biochem. Biophys.* **95,** 271.
[16] SMITH, L. (1955) in *Methods in Enzymology* (COLOWICK, S. P. and KAPLAN, N. O., eds.), Vol. II, p. 732, Academic Press, New York.
[17] LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, R. J. (1951) *J. Biol. Chem.* **193,** 265.
[18] WADDEL, W. J. (1956) *J. Lab. Clin. Med.* **48,** 311.