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PROTEIN DEGRADATION AND PROTEOLYTIC ACTIVITY IN THE COTYLEDONS OF GERMINATING PEA SEEDS (*PISUM SATIVUM*)

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Abstract—A study has been made of the redistribution of nitrogenous compounds during the germination of peas. The rapid translocation of nitrogen from the reserve proteins in the cotyledons to the growing axis appears to involve the degradation of proteins to amino acids. Studies have been made of enzymes capable of effecting such hydrolyses and four enzymes with proteolytic activity have been demonstrated. The characteristics of these enzymes are described and their possible physiological role discussed.

INTRODUCTION

THE GERMINATION and post-germination of seeds is characterized by the mobilization of reserves from the storage tissues and the transfer of solubilized derivatives to the growing embryo axis. One of the major reserves in legume seeds is protein, and in peas this component can make up about 30 per cent of the dry weight. The protein reserve exists in two forms—vicilin and legumin[1] located in the protein bodies.[2] During germination this protein reserve is depleted and nitrogen accumulates in the developing axis. The transfer of nitrogen is apparently achieved by release of amino acids from the reserve proteins by hydrolyses, and their subsequent translocation to the developing axis.

While these generalities are well known, the mechanism by which the proteins are converted to amino acids is less clearly understood. It is generally assumed that the hydrolysis is affected by various proteolytic enzymes which develop during germination. However, evidence for the existence and operation of such enzymes is meagre.

Soedigo and Gruber[3] demonstrated a proteolytic enzyme in pea seeds which hydrolyzed casein at pH 8·0, but no physiological role was assigned to this enzyme. Mergentine *et al.*[4] showed that the caseolytic activity of extracts from peas was optimal at pH 5·5. Using a gelatine substrate, Danielsson[5] demonstrated optimal proteolytic activity in extracts of pea seedlings at pH 6·7. It was claimed that proteolytic activity was greatest in extracts of developing, as opposed to germinating, peas.[5] Young and Varner[6] and more recently Henshall and Goodwin[7] have demonstrated apparent caseolytic activity in extracts from

[1] C. E. DANIELSSON, *Acta Chem. Scand.* **4**, 762 (1950).
[2] J. E. VARNER and G. SCHIDLOVSKY, *Plant Physiol.* **38**, 139 (1963).
[3] R. SOEDIGO and M. GRUBER, *Biochem. Biophys. Acta* **44**, 315 (1960).
[4] M. MERGENTINE and E. H. WIEGAND, *Fruit Products J.* **26**, 72 (1946).
[5] C. E. DANIELSSON, *Acta Chem. Scand.* **5**, 541 (1951).
[6] J. L. YOUNG and J. E. VARNER, *Arch. Biochem. Biophys.* **84**, 71 (1959).
[7] J. D. HENSHALL and T. W. GOODWIN, *Photochem. Photobiol.* **3**, 243 (1964).

the cotyledons of germinating peas at pH 7·0. These workers demonstrated only small changes in proteolytic activity as germination proceeded. In contrast, Irving and Fontaine[8] showed that the proteases of peanut cotyledons increased during germination and had optimal activity at pH 8·0. During germination of *Cucurbita maxima* there is a marked increase of protease activity in the cotyledons through the third day, followed by a decline to a low level.[9]

These contrasting findings, and the observation that protease activity in extracts from cotyledons of germinating peas showed little relationship to protein mobilization, prompted the more detailed investigation into proteolytic activity of pea cotyledons presented in this paper.

RESULTS

1. *Changes in Nitrogen Content*

Characteristically it was found that as germination progressed there was a depletion of total nitrogen in the cotyledons with an accumulation of this component in the growing axis (Fig. 1). The decline in total cotyledonary nitrogen was most rapid between the second and

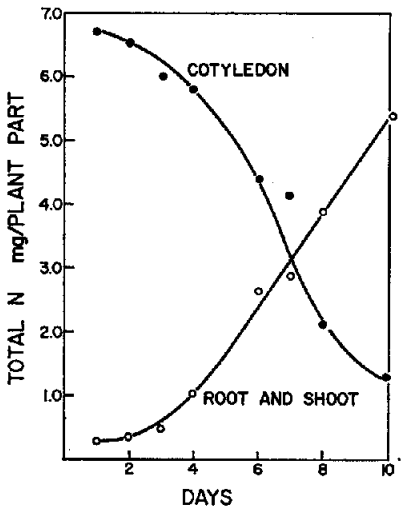

FIG. 1. CHANGES IN THE DISTRIBUTION OF TOTAL NITROGEN DURING THE GERMINATION OF PEA SEEDLINGS.

eighth days. While the total nitrogen of the cotyledons decreased it was found that the alcohol-soluble and α-amino nitrogen was increasing (Fig. 2). Furthermore the alcohol-soluble amino nitrogen content of the axis also increased rapidly over the germination period. These observations are consistent with the suggestion that cotyledonary protein is hydrolyzed to amino acids which are then transported to the developing axis.

[8] G. W. IRVING and T. D. FONTAINE, *Arch. Biochem.* **6**, 351 (1945).
[9] L. WILEY and F. W. ASHTON, *Physiol. Plantarum* **20**, 688 (1967).

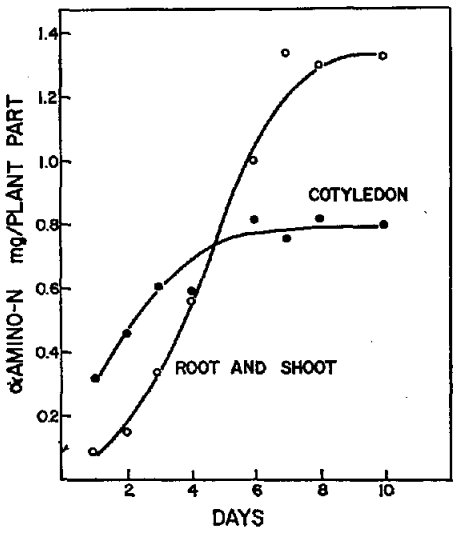

FIG. 2. THE DISTRIBUTION OF α-AMINO NITROGEN IN GERMINATING PEA SEEDLINGS.

2. *Protease Activity*

By the use of dialyzed extracts of pea cotyledons (see Methods) it was possible to demonstrate that there were apparently two caseolytic systems (Fig. 3). One had a pH optimum of 5·5 (0·2 M citrate) and the other had a pH optimum of 7·0 (0·2 M phosphate).

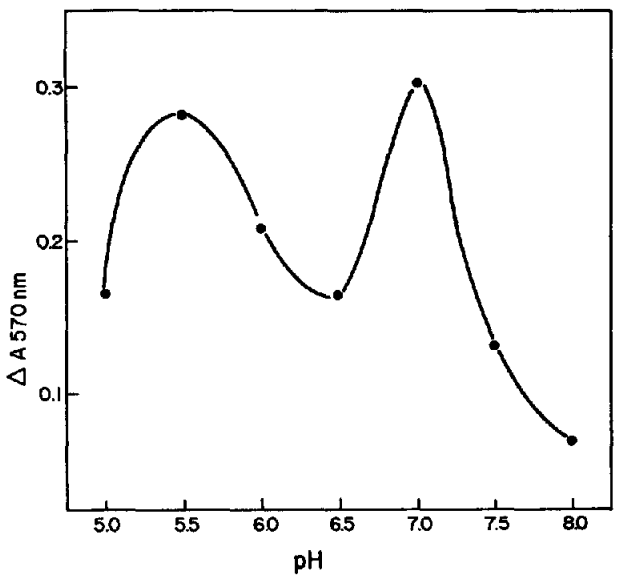

FIG. 3. THE INFLUENCE OF pH ON PROTEOLYTIC ACTIVITY IN EXTRACTS OF COTYLEDONS FROM GERMINATING PEA SEEDLINGS.
pH 5·0–pH 6·0: 0·2 M sodium citrate
pH 6·5–pH 8·0: 0·2 M sodium phosphate.

The release of α-amino nitrogen from the casein substrate was initially slow (Fig. 4) but increased with time. This observation might indicate that the enzyme(s) being assayed was a non-specific exopeptidase and the low initial rate of α-amino nitrogen release might be related to a lack of available free ends of molecules.

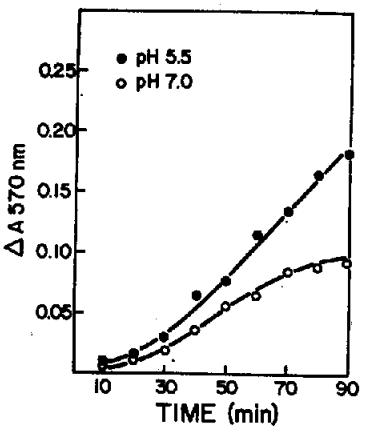

FIG. 4. THE EFFECT OF INCUBATION TIME ON THE RELEASE OF α-AMINO NITROGEN FROM CASEINE BY EXTRACTS OF PEA COTYLEDONS.

Release of α-amino nitrogen from the casein substrate was linearly related to the quantity of enzyme added.

3. *Changes in Proteolytic Activity with Age*

In view of the apparent operation of two proteolytic systems it was of interest to determine if there were changes in enzyme levels during the course of germination. Assays of the proteolytic activity of extracts of cotyledons of various ages indicated that the activity of both the pH 5·5 and pH 7·0 enzymes showed similar patterns of development (Fig. 5). Initially activity was low and increased as germination proceeded. It should be noted that while both pH 5·5 and 7·0 proteolytic activity showed similar patterns of development the most rapid increase in activity occurred *after* the most rapid depletion of cotyledonary nitrogen.

4. *Peptidase Activity*

The synthetic peptides leucine *p*-nitroanilide (LPA) and α-benzoyl-DL-arginine-*p*-nitroanilide (BAPA) were hydrolyzed by pea cotyledon extracts. LPA was hydrolyzed most rapidly at pH 8·0 and BAPA was hydrolyzed by the dialyzed pea extracts over a wider range of pH with optimal activity between pH 6·5 and 8·0 (Fig. 6). In routine assays the incubation for BAPA hydrolyses was carried out at pH 7·0. The enzymatic release of *p*-nitroaniline from LPA and BAPA was linear over the time periods studied. However, in view of the much greater LPAase activity in the pea extracts, LPA hydrolysis was determined at 5 min and BAPase activity at 15 or 30 min. Release of *p*-nitroaniline from the substrates was linear over a wide range of enzyme concentrations, however, because of the turbidity of the dialyzed pea extracts relatively low enzyme concentrations were used in the routine assays.

In experiments in which the substrate concentrations were varied it appeared that the concentration of BAPA used was limiting (Fig. 7). Thus each increment of substrate gave an

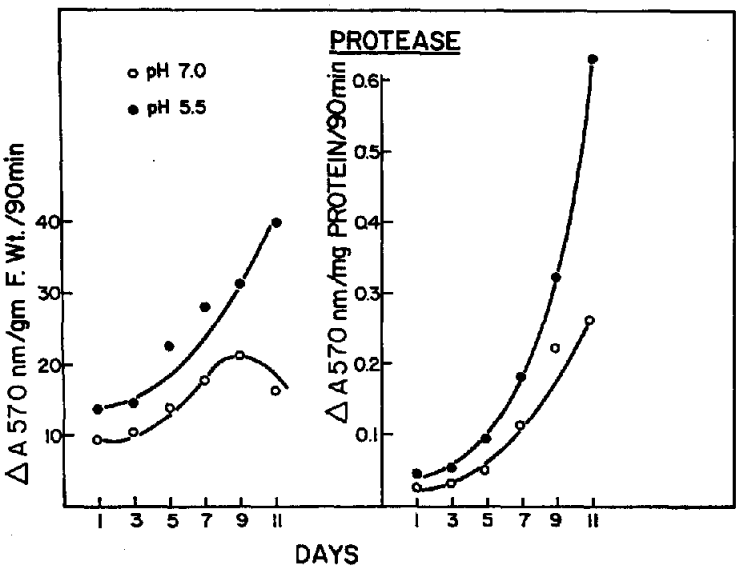

FIG. 5. CHANGES IN PROTEOLYTIC ACTIVITY IN EXTRACTS OF PEA COTYLEDONS DURING GERMINATION.

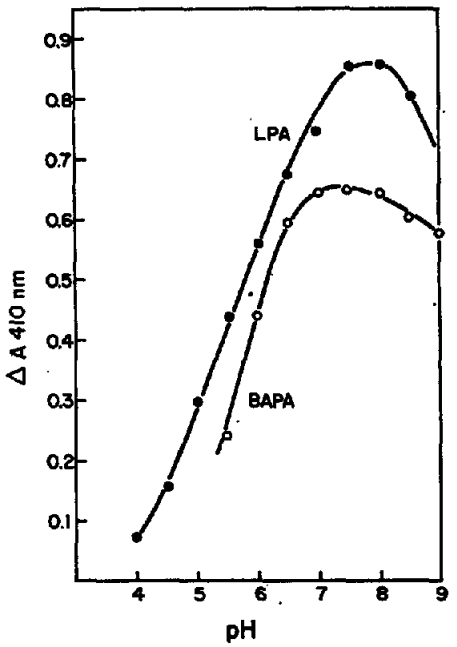

FIG. 6. THE INFLUENCE OF pH ON THE CAPACITY OF EXTRACTS OF PEA COTYLEDONS TO HYDROLYZE L-LEUCINE-*p*-NITROANILIDE (LPA) AND α-BENZOYL-DL-ARGININE-*p*-NITROANILIDE (BAPA).

increased production of *p*-nitroaniline following incubation with enzyme. Saturating concentrations of substrate could not be used due to the low solubility of α-benzoyl-DL-arginine-*p*-nitroanilide. LPA concentrations used routinely in the assays were sufficient to saturate the enzyme (Fig. 7).

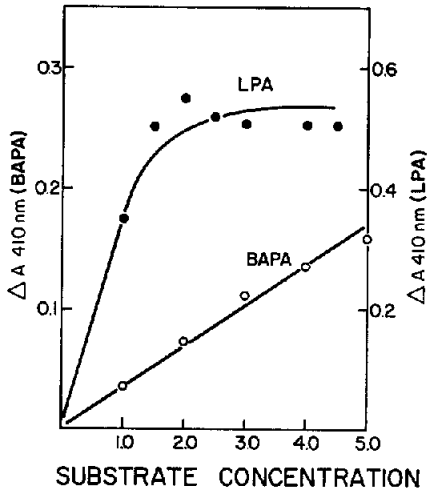

FIG. 7. THE INFLUENCE OF VARIOUS SUBSTRATE CONCENTRATIONS ON THE CAPACITY OF EXTRACTS TO RELEASE *p*-NITROANILIDE. Substrate concentrations were varied by adding varying volumes of stock solutions to 0·2 M phosphate buffer to give a final substrate volume of 5 ml.

5. *Changes in Peptidase Activity with Age*

Assays of the peptidase activity of extracts prepared from the cotyledons of pea seedlings of various ages indicated that the activity of both LPAase and BAPAase was initially high and declined as germination progressed (Fig. 8) on a per g. fresh weight basis. On a specific activity basis it was found that LPAase activity increased following germination. BAPAase activity on a per mg protein basis remains relatively constant throughout the germination period. It is probable that this represents a slower turnover of LPAase rather than indicating a synthesis of the enzyme as germination proceeds.

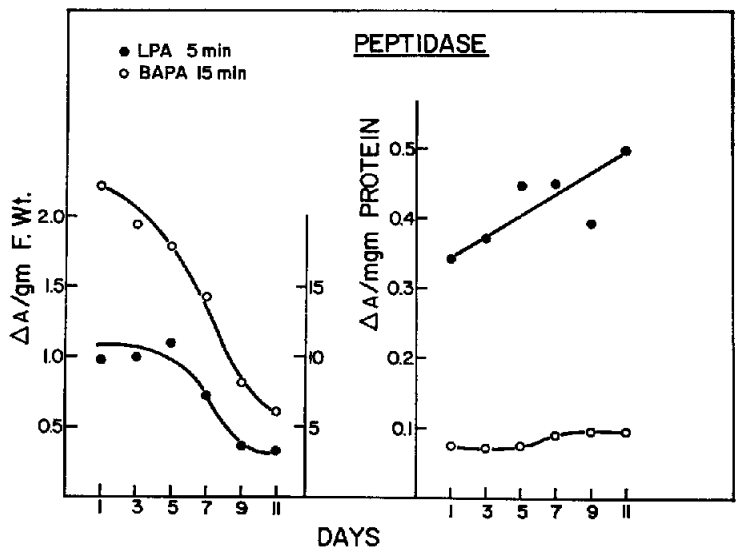

FIG. 8. CHANGES IN PEPTIDASE ACTIVITY IN EXTRACTS OF PEA COTYLEDONS FOLLOWING GERMINATION.

DISCUSSION

During the germination of pea seeds there is a depletion of protein nitrogen from the cotyledons. The accumulation of free amino nitrogen during this phase indicates that the degradation of proteins is probably accomplished by the activity of proteolytic enzymes.

Using dialyzed buffered extracts of pea cotyledons it has been possible to demonstrate the release of TCA-soluble amino nitrogen from a caseine substrate, indicating the existence of proteolytic enzymes. The observation that proteolytic activity was optimal at pH 5·5 and pH 7·0 suggests that at least two proteolytic enzymes occur in the cotyledons.

However, the physiological role of the respective proteases is not apparent. Foreseeably they may preferentially hydrolyze the two main protein reserves legumin and vicilin. The observation that proteolytic activity increased as germination proceeds and as protein decreases in the cotyledons is consistent with this suggestion. However, the most rapid protein loss from the cotyledons occurs between the third and eighth day following germination (Fig. 1) whereas the peak of proteolytic activity is not reached until much later (Fig. 5).

The observed increase in proteolytic activity as germination proceeds is at variance with the observations of other workers[2, 7] but agrees with the findings of Irving and Fontaine[8] with peanuts and those of Wiley and Ashton[9] with cucumber. Some of this disparity might be related to the method of enzyme assay. Investigations in our laboratory have indicated that there is little relationship between changes in absorptivity at 280 nm and the accumulation of amino nitrogen. Crude plant extracts contain a variety of compounds which absorb light in the u.v. region and changes in the concentration of these constituents during a 90 min incubation at 40° would obviously contribute to changes in absorbance at 280 nm unrelated to proteolytic acitivity.

The observations that the synthetic peptides LPA and BAPA were readily hydrolyzed by the extracts of pea cotyledons indicates the operation of active peptidases. LPA is reported to be a specific substrate for leucine amino peptidase and thus the rapid hydrolyzes of this substrate presumably indicates a high activity of this enzyme.

It has been suggested[10] that BAPA is a substrate for trypsin type enzymes. However, the pattern of development of caseolytic (trypsin type) activity in the germinating seeds is completely different from that recorded for BAPAase activity. Similar divergences can be noted in the barley endosperm. Jacobsen and Varner[11] demonstrated that the capacity to hydrolyze gliadin by barley aleurone cell extracts was initially low and increased as germination progressed or following treatment with gibberellic acid. In contrast Burger[12] demonstrated that BAPAase did not follow any particular pattern during germination and did not increase in response to gibberellic acid. Thus the pattern of development of peptidase and proteolytic activity in peas appears to be similar to that in barley. Initially peptidase activity is high and proteolytic activity is low, only at later stages does proteolytic activity develop.

The physiological role of the peptidases remains to be established, they may be more closely involved with the mobilization of reserve proteins than the proteolytic enzymes. In this respect, peptidase activity is high during the period of maximal protein depletion of the cotyledon and in other experiments in our laboratory we have been unable to detect appreciable peptidase activity in senescing leaf discs although appreciable caseolytic activity is present. In this context then, peptidases might have activity associated with the utilization of reserve proteins whereas the caseolytic enzymes might be associated with protein degradation and turnover associated with growth and development.

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[11] J. V. Jacobsen and J. E. Varner, *Plant Physiol.* **42**, 1596 (1967).
[12] W. C. Burger and H. W. Siegelman, *Physiol. Plantarum* **19**, 1089 (1966).

MATERIALS AND METHODS

Pea seeds (*Pisum sativum* var. *Burpeeana*) were sown in moist vermiculite (2 vermiculite:1 vol. water) and maintained in a dark germinator at 28°. At intervals as required the seedlings were harvested and used in the various analyses and assays.

Total nitrogen and alcohol-soluble amino nitrogen were determined as described previously.[13]

Standard protease assay 10 g of pea cotyledons were homogenized in 30 ml of grinding mix consisting of 0·05 M Tris containing 0·005 M cysteine hydrochloride adjusted to pH 7·5 for 2 min at high speed in a Virtis homogenizer. The resulting homogenate was filtered through cheese-cloth and then centrifuged at 12,000 *g* for 20 min. The supernatant was then dialyzed against four changes of 0·01 M phosphate buffer, pH 7·0, for 24 hr. After this time the extract was recentrifuged at 12,000 *g* to remove material precipitated during the dialysis. The supernatant which was still slightly cloudy was used as enzyme source.

In the routine protease assay 1 ml of this extract was incubated with 1 ml of 1 per cent casein and 1 ml of 0·2 M buffer, pH 5·5 (citrate), or pH 7·0 (phosphate). The mixture was incubated at 40° for 90 min after which time the reaction was terminated by the addition of 1 ml of 20 per cent TCA. The precipitated material was removed by centrifugation at 3000 *g* for 15 min and the cleared supernatant used for the determination of amino nitrogen. Usually 0·1 ml of supernatant was sufficient for the determination of amino nitrogen by the ninhydrin procedure described by Yemm and Cocking.[14]

Peptidase assays were performed by modification of the methods described by Erlanger *et al.*[10] 5×10^{-4} M solutions of the synthetic peptides were prepared by allowing the material to dissolve overnight in 0·2 M phosphate buffer of appropriate pH in a shaking water-bath at 35°.

Five ml of the LPA substrate solutions were incubated with 0·1 ml. of pea cotyledon extract and the change in absorptivity at 410 nm which occurred during a 5 min incubation at 40° was recorded. The reaction was initiated by addition of enzyme.

For BAPAase assay 5 ml of stock substrate was incubated with 0·1 ml of dialyzed extract for 15 min and the release of *p*-nitroanilide recorded by measuring the increase in A at 410 nm.

Source of Substrates

The 1 per cent caseine solution was prepared by suspending 1 g of casein (Mann Research Laboratories high nitrogen) in 50 ml of 0·01 N NaOH. After dispersion the pH was adjusted to 7·0 by the slow addition of 0·1 N HCl and the solution made to volume.

L-Leucine-*p*-nitroanilide (LPA) and α-benzoyl-DL-arginine-*p*-nitroanilide (BAPA) used for peptidase assay were supplied by Nutritional Biochemicals Corporation, Cleveland, Ohio.

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