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# Suborganellar Localization of Proteinase Catalyzing the Limited Hydrolysis of Pumpkin Globulin[1]

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## ABSTRACT

**Protein bodies were prepared from the cotyledons of pumpkin (*Cucurbita* sp.) seeds by employing a nonaqueous isolation method. Both light micrographic examination and the marker enzyme assays have shown that the isolated protein bodies were intact and contamination with other cell organelles or cytoplasmic components was negligible. A proteolytic enzyme catalyzing the limited hydrolysis of carboxymethylated γ′ chain of globulin was found to be present in the protein bodies. The specific activity in the protein body (18 units per milligram protein) was higher than that in the whole cell extract (13 units per milligram protein), indicating that the limited proteolytic enzyme was localized in the protein body.**

**After lysis of the protein bodies using hypotonic buffer solution, the suborganellar components (matrix, membranes, and crystalloids) were separated by sucrose density gradient centrifugation. The crystalloid was composed of only globulin, a major seed protein. The major proteins of matrix and membrane fractions were shown to have mol wt of approximately 10,000. About 90% of the limited proteolytic activity was found in the matrix region.**

When seeds germinate, storage materials associated with protein bodies are hydrolyzed by the action of many enzymes. Since the storage protein of pumpkin (*Cucurbita* sp.) seeds is an 11S type globulin, comprising more than 80% of the total protein content in the dry seeds (9), the cotyledon is a simpler material to study the breakdown of the storage protein than most other seeds containing various kinds of storage proteins.

Each subunit of the pumpkin globulin is composed of an acidic polypeptide (mol wt, 34,000 or 36,000) and a basic polypeptide (mol wt, 22,000) linked by a disulfide bond (9). The detailed chemical properties of the protein have been reported previously (7, 15). The globulin molecules start to disappear at an early stage of germination and about 90% is degraded in 4 d (8). During this period, a limited proteolytic product, $F_{\alpha\beta}$,[2] accumulates, which is eventually hydrolyzed to smaller peptides and amino acids (8). Some properties of the enzyme carrying out limited proteolytic hydrolysis of the acidic constituent (γ′ chain of the globulin) have been reported previously (4). This enzyme exhibited high substrate specificity. It can hydrolyze the γ′ chain of the globulin efficiently, but cannot hydrolyze the basic chain of the globulin or several animal proteins (BSA, ovalbumin, Cyt *c*) (4). This enzyme is thought to play an important role in the degradation of the globulin. Therefore, to clarify the mechanism of the degradation of storage protein, globulin, the characterization of the limited proteolytic enzyme is urgently necessary. In this communication, the intracellular and suborganellar localizations of the limited proteolytic enzyme in dry pumpkin seeds were examined.

## MATERIALS AND METHODS

**Protein Body.** Protein bodies were isolated from dry pumpkin (*Cucurbita* sp., hybrid, Tetsukabuto-Nankin) seeds essentially following the method reported by Yatsu and Jacks (18, 19), inasmuch as they disintegrate in some aqueous media. After removal of the hard seed coat and inner thin testa, 5 g of cotyledons were homogenized in a blender using 50 ml of glycerol. Removing the inner thin testa was necessary because this tissue was found to possess high catalase activity (per fresh weight basis) and its fragments pelleted with the protein bodies. The resulting homogenate was strained through cheesecloth and centrifuged at 13,000*g* for 10 min. The pellet was resuspended in 50 ml glycerol and centrifuged again at 13,000*g* for 10 min to yield the protein body fraction. All procedures were carried out at room temperature. A longer centrifugation time resulted in increased contamination of the protein body fraction.

**Marker Enzyme Assays.** All enzyme assays except acid phosphatase and phosphodiesterase were carried out at room temperature of about 20°C.

Glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase were assayed in 1 ml reaction mixture, containing 0.3 mM glucose-6-P or 0.2 mM 6-P-gluconate, 25 mM Tris-HCl buffer (pH 7.5), 10 mM $MgCl_2$, 0.17 mM $NADP^+$, and diluted enzyme solution. The absorption increase was measured at 340 nm.

Acid phosphatase and phosphodiesterase were assayed essentially following the method of Nishimura and Beevers (13), employing *p*-nitrophenylphosphate (5 mM) and bis(4-nitrophenyl)phosphate (5.6 mM) as substrates, respectively. Assays were performed in a 0.5 ml reaction volume, containing the substrate, 0.1 M Na-acetate buffer (pH 5.5), and diluted enzyme solution. After incubating at 37°C for 10 min, the reaction was stopped by adding 2.5 ml of 0.2 M NaOH. The amount of *p*-nitrophenol liberated was measured at 400 nm.

The assays of catalase (11), NAD malate dehydrogenase (1), and fumarase (16) followed those described in the literatures.

**Assay for Limited Proteolytic Activity.** Cm-γ′ chain used as substrate was prepared from carboxymethylated globulin according to the procedures reported previously (9). Proteolytic activity was measured by the loss in intensity of the band due to Cm-γ′ chain after separation of the polypeptides by SDS-gel electropho-

[1] This is paper VIII in the series "Pumpkin (*Cucurbita* sp.) seed globulin."

[2] Abbreviations: $F_{\alpha\beta}$, limited proteolytic product of globulin; γ′ chain, constituent acidic polypeptide chain of globulin (mol wt, 34,000); Cm, carboxymethylated.

resis. The reaction mixture contained the following components in a total volume of 115 μl: 0.1 M Na-acetate buffer (pH 5.5), 0.1 mg of Cm-γ′ chain, and enzyme solution. The reaction was carried out at 37°C for 3 to 6 h. Just before the reaction was terminated with 0.1 ml 20% TCA, 5 μl of 1% BSA was added as a marker protein for correcting the loss of protein during the subsequent treatments; BSA is resistant to attack by the proteolytic enzyme (4). After acidification, the precipitate was collected by centrifugation and washed with acetone, then dissolved in 0.1 ml 25 mM Tris-acetate buffer (pH 8.2) containing 1% SDS and finally analyzed by SDS-gel electrophoresis. After destaining, the gel slabs were scanned at 570 nm with a Gelman Densitometer, type ACD-18. Intensity of the band of Cm-γ′ chain after incubation ($S_{\gamma,t}$) was corrected by intensity of the BSA band ($S_{BSA,t}$). Activity was calculated by the equation:

$$\frac{100}{t} \times \left(1 - \frac{S_{\gamma,t}/S_{BSA,t}}{S_{\gamma,0}/S_{BSA,0}}\right),$$

$t$ representing the incubation time (h). One unit of the activity was defined as 1 μg of Cm-γ′ chain cleaved per h.

**Sucrose Density Gradient Centrifugation.** Fractionation of protein bodies was performed by sucrose density gradient centrifugation essentially as described by Youle and Huang (20). Glycerol-isolated protein bodies (23 mg protein) were lysed by the addition of 0.5 ml 20 mM Na-acetate buffer (pH 5.5), and 0.2 ml of the ruptured fraction was layered on a sucrose gradient consisting of 1 ml cushion of 68% (w/w) sucrose, 9 ml sucrose solution increasing linearly from 30% to 68% sucrose, 2 ml of 15% sucrose, and 2 ml of 5% sucrose in a 17-ml tube. The gradient was centrifuged for 6.5 h at 21,000 rpm in a Beckman model L2-65B centrifuge using a Spinco SW 25.3 rotor at 4°C. After centrifugation, fractions (1.2 ml) were collected by an ISCO gradient fractionator (model 640), and the protein content was analyzed using Bio-Rad Protein Assay Kit using bovine gamma globulin as a standard.

**Light Photomicrograph.** Transverse tissue sections from the central intervein region of dry seed were fixed with 2% glutaraldehyde in 50 ml cacodylate buffer (pH 6.7) at room temperature for 1 h, followed by washing with the same buffer for 1 h. Sections 5 μm thick were cut with a Leiz-Histokryotom. Sections were observed with a differential interference microscope, Olympus BHS-N. Glycerol-isolated protein bodies were observed with an Olympus BHA microscope.

**SDS-Polyacrylamide Gel Electrophoresis.** TCA-insoluble fractions were washed with acetone, dissolved in 25 mM Tris-acetate buffer (pH 8.2) containing 1% SDS (about 1 mg protein/ml), and subjected to SDS-gel electrophoresis using 7.5% gel slab. Other experimental conditions employed were the same as described in the preceding paper (9).

## RESULTS AND DISCUSSION

**Isolation of Protein Bodies.** Photomicrograph of the cotyledon of dry pumpkin seed given in Figure 1A shows the presence of many protein bodies. The preparation of isolated protein bodies were examined under the light microscope and Figure 1B clearly shows the presence of globoids, indicating that the isolated protein bodies appeared essentially similar as observed by the light microscope in Figure 1A.

In order to assess the purity of the isolated protein bodies, activities of several marker enzymes were measured and results are given in Table I. In the table, the specific activities of each enzyme in glycerol-isolated protein body fraction and whole cell extract, and also the ratio of these two specific activities, are presented. Marker enzymes assayed were: catalase (glyoxysomes), NAD-malate dehydrogenase (glyoxysomes, mitochondria, cytosol), glucose-6-P dehydrogenase and 6-P-gluconate dehydrogenase (plastids, cytosol), and fumarase (mitochondria).

As shown in Table I, glucose-6-P dehydrogenase, 6-P-gluconate dehydrogenase and fumarase were not detectable in the protein

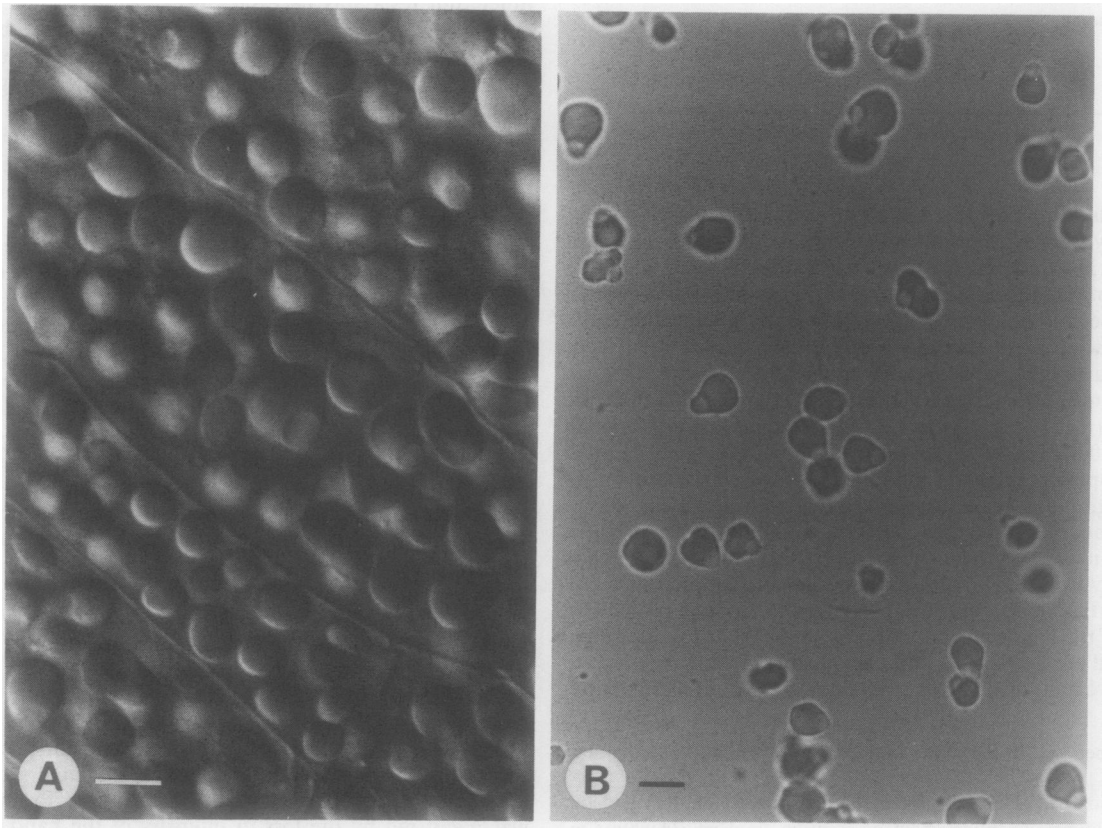


FIG. 1. Light photomicrographs of pumpkin cotyledon cells (A) and glycerol-isolated protein bodies from dry seeds (B). Bars, 10 μm.

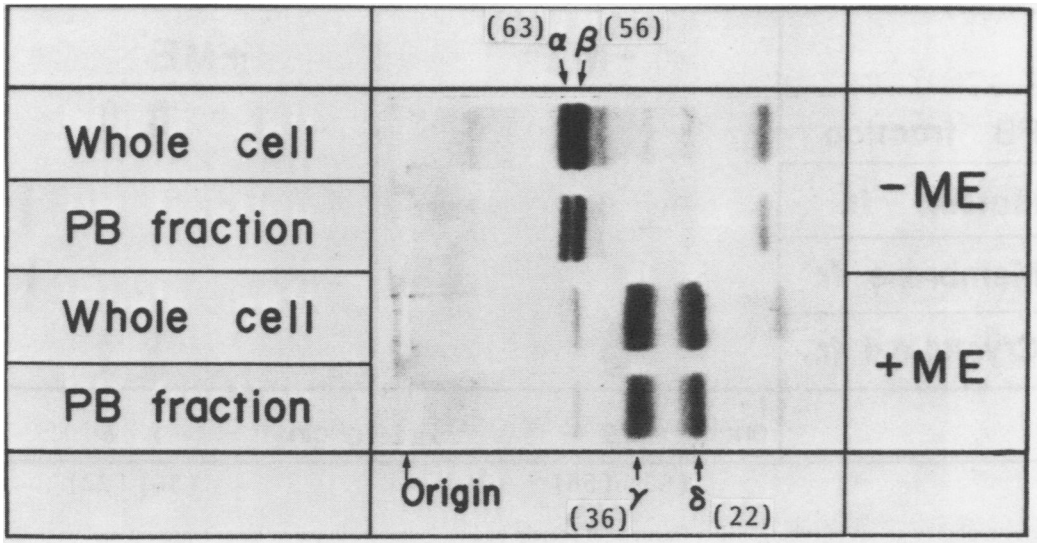


FIG. 2. SDS-gel electrophoretograms of the glycerol-isolated protein bodies shown in Figure 1B and whole cell extract. Numbers in parentheses represent mol wt in thousands. ME and PB represent β-mercaptoethanol and protein body.

Table I. *Limited Proteolytic Activities and Marker Enzyme Activities in Whole Cell Extract and Glycerol-Isolated Protein Body Fraction*

| | Specific Activity[a] | | |
|---|---|---|---|
| | Whole cell (W) | Protein body fraction (PB) | PB/W |
| | *Units/mg protein* | | % |
| Limited proteolytic activity[b] | 13 | 18 | 140 |
| Catalase[c] | $2.05 \times 10^3$ | $2.40 \times 10^2$ | ×11.7 |
| Malate dehydrogenase[c] | $2.41 \times 10^2$ | 14.0 | 5.8 |
| Glucose-6-P dehydrogenase[c] | 0.41 | <0.01 | |
| Gluconate-6-P dehydrogenase | 1.13 | <0.01 | |
| Fumarase[c] | 0.27 | <0.01 | |
| Acid phosphatase[c] | 10.1 | 6.03 | 59.7 |
| Phosphodiesterase[c] | 3.04 | 1.40 | 46.1 |

[a] Protein was analyzed by the method of Lowry (10) using BSA as standard.

[b] One unit was defined as 1 μg of Cm-γ′ chain cleaved per h.

[c] One unit was defined as 1 nmol product produced per min.

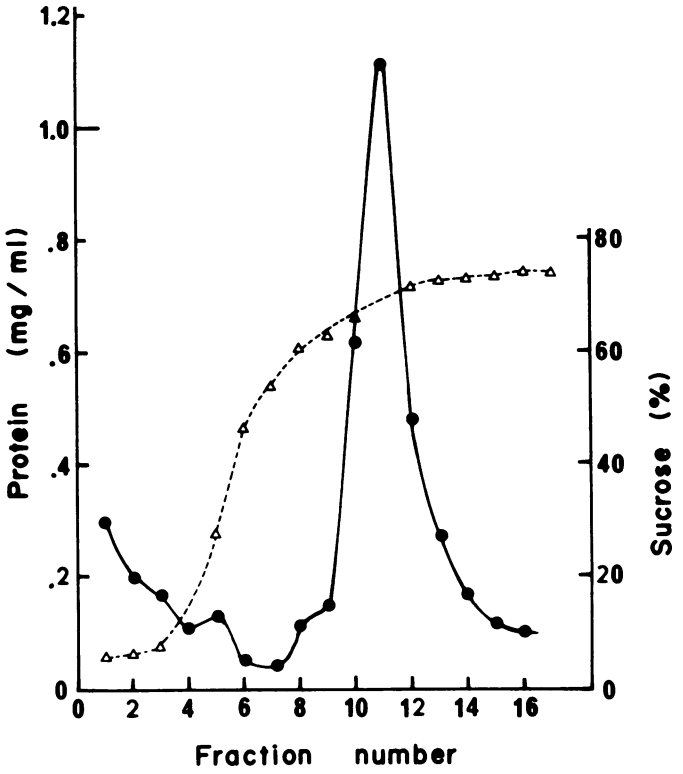


FIG. 3. Separation of suborganellar inclusions from lysed protein bodies on sucrose density gradient centrifugation.

body fraction. The absence of these enzymes suggests that the protein body fraction is not contaminated by cytoplasmic, plastid, or mitochondrial fractions. NAD-malate dehydrogenase (5.8%) and catalase (11.7%) specific activities in the protein body fraction were less than those in the whole cell extract. It has been reported that NAD-malate dehydrogenase is present in the protein bodies isolated from castor bean (12). Catalase activity detectable in the protein body fraction can be ascribed to the contamination by fragments of the testa or glyoxysomes. Specific activities of acid phosphatase and phosphodiesterase in the protein body fraction were approximately one-half of those in the whole cell extract. It has been reported that these two enzyme activities are present in the glycerol-isolated protein body fraction of castor bean (12).

Both the whole cell extract and the protein body fraction were subjected to the SDS-gel electrophoresis to examine the protein constituents (Fig. 2). The proteins of the whole cell extract were resolved into several components having different mol wt, the gel pattern resembling that of the protein body fraction. Such resemblance was predicted, inasmuch as the majority of the cellular proteins is localized in the protein bodies. Without β-mercaptoethanol treatment, two main bands which are attributable to the constituent α and β subunits of globulin molecule and the minor band with mol wt around 10,000 were detected. By the addition of β-mercaptoethanol, we were able to detect major bands presumably due to the reduced polypeptide chains, γ(γ′) and δ. It is conceivable that in addition to globulin, smaller protein species with mol wt around 10,000 are derived from the protein body.

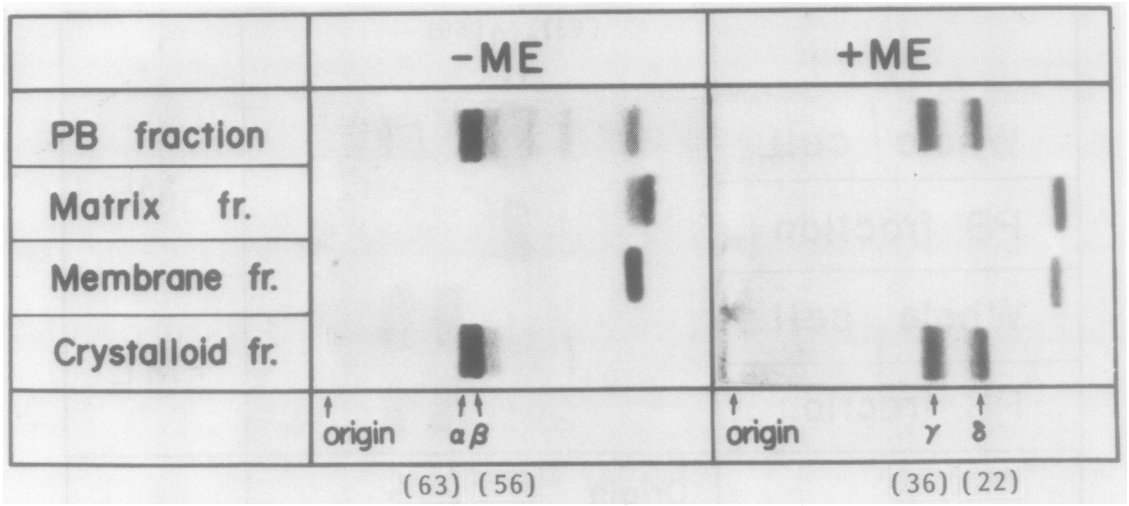


FIG. 4. SDS-gel electrophoretograms of suborganellar fractions of protein bodies. Numbers in parentheses represent mol wt in thousands. ME and PB represent β-mercaptoethanol and protein body.

Table II. *Limited Proteolytic Activities in Suborganellar Fractions of Protein Bodies*

One unit of the activity was defined as 1 μg of Cm-γ′ chain cleaved per h.

| | Specific Activity | Total Activity |
|---|---|---|
| | *unit/mg protein* | % |
| Matrix fraction | 230 | 87.0 |
| Membrane fraction | 40 | 6.7 |
| Crystalloid fraction | 4.4 | 7.0 |

**Presence of Limited Proteolytic Activity Against Cm-γ′ Chain in Isolated Protein Bodies.** Pumpkin seeds contain enzymatic activity that can hydrolyze the acidic γ′ polypeptide chain, but not the basic δ chain, of the globulin (4). To clarify the localization of the limited proteolytic activity, the specific activities in the protein body fraction and the whole cell extract were measured using Cm-γ′ chain as a substrate. As shown in Table I, the specific activity in the protein body fraction (18 units/mg protein) is higher than that in the whole cell extract (13 units/mg protein), suggesting that the limited proteolytic activity is localized in the protein bodies.

**Separation of Suborganellar Inclusions from Lysed Protein Bodies.** It was shown that a protein body enclosed by the membrane contains additional inclusions of a protein crystalloid and a phytin globoid embedded in proteinaceous matrix (6). These suborganellar components can be separated by the gradient centrifugation of the lysed preparation of protein bodies. Three fractions, *i.e.* (a) supernatant, (b) density 1.12 g/ml [27.5% (w/w) sucrose] fraction, and (c) density 1.34 g/ml [68.8% (w/w) sucrose] fraction are separated (Fig. 3). The water-soluble matrix proteins, remaining on top of the sucrose gradient were found to represent 15% of the total protein of the protein bodies.

The second fraction (density, 1.12 g/ml) formed a single reproducible band in the gradient. Because this band is clearly visible but contains low protein content, it appeared to be the membrane of the protein bodies. Youle and Huang (20) have reported that a fraction with a similar density (1.15 g/ml) separated from castor bean protein bodies on a sucrose density centrifugation is the membrane of the protein bodies.

The third fraction (density, 1.34 g/ml) was found to contain more than 80% of the total protein of the protein body fraction. The crystalloid protein was insoluble in water but soluble in saline solution, and thus, can be categorized as globulin.

According to the results of Youle and Huang (20) on castor bean globoid, it is expected that globoid would be found at the bottom of the sucrose gradient. However, analyses for the globoid fraction were not made.

**Protein Components of Matrix, Membrane and Crystalloid of the Protein Bodies.** Protein components of the three suborganellar fractions were analyzed by the SDS-gel electrophoresis (Fig. 4). The protein band pattern of the crystalloid fraction indicates that the fraction consists of only globulin. On the other hand, the smaller protein species with mol wt about 10,000 were found to be present in both matrix and membrane fractions. Inasmuch as the protein content is low in these two fractions, concentrated samples were applied to the SDS-gel electrophoresis. However, they did not give any extraneous bands of the larger mol wt. The profile of the protein constituent was basically analogous with those reported by Youle and Huang (20, 21), except that pumpkin seed protein contains neither phytohemagglutinin nor ricin observed in the matrix proteins of castor bean.

**Limited Proteolysis of Cm-γ′ Chain by Suborganellar Fractions of Protein Bodies.** As presented in Table II, the specific proteolytic activities were determined among three suborganellar fractions, *i.e.* matrix, membrane and crystalloid, and the highest activity was found to reside in the matrix fraction, nearly all activities being present in this fraction (87%). On the other hand, in spite of the fact that more than 80% of the total protein of protein body is present in the crystalloid fraction, this fraction contained only 6.3% of the total activity detectable in the protein body fraction. It is thus likely that the limited proteolytic activity is localized in the matrix region of the protein bodies of the dry pumpkin seeds.

Results of ultrastructural investigations show that during the step of globulin degradation the crystalloids are broken down from their periphery (6). Immunofluorescent conjugation examination has shown that the polypeptides reactive with the antibody against the globulin are localized in the matrix region (6), indicating that the insoluble globulin molecule is presumably converted to the more soluble form liable to be the subsequent proteolysis by the attack of the proteolytic enzyme present in the matrix region. This implication appears to be consistent with our result concerning the localization of the limited proteolytic enzyme, because the limited proteolytic product $F_{\alpha\beta}$ is more soluble than the globulin (8), and $F_{\alpha\beta}$ being readily diffusible into the matrix. Because the limited proteolytic enzyme cannot hydrolyze $F_{\alpha\beta}$, $F_{\alpha\beta}$ produced might be further degraded in the matrix of the protein bodies by other enzyme activities, and several proteolytic enzyme activities have been reported in germinating pumpkin cotyledon (5).

Chemical properties of the pumpkin seed globulin are found to

be similar 11S globulins of other plant origins (3). It was observed that 11S globulins of pumpkin (17) and castor bean (14) were broken down through limited proteolytic product with similar mol wt to that of $F_{\alpha\beta}$. Bul'maga and Shutov (2) purified endopeptidase hydrolyzing the native legumin (11S globulin) and vicilin (7S globulin) from the germinating vetch seeds. However, the 11S globulin of vetch seeds is not hydrolyzed to produce an intermediate such as $F_{\alpha\beta}$ by the purified endopeptidase.

Further purification of the limited proteolytic enzyme from the dry pumpkin seeds and the detailed analytical studies on the enzyme properties may provide us more useful information concerning the mechanism of 11S globulin degradation during germination.

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