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Role of Proteinases in the Degradation of the Major Storage Proteins of Linseed Germinating Seeds

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دور أنزيمات التحلل البروتيني في هدم البروتينات المخزنة في بذور الكتان النامية

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وجد أن إنزيمات التحلل البروتيني التي تعمل على الكازين والأذوكول والجلوبيلين المستخلص من الكتان تزداد من بدء الإنبات حتى مرور ٤٨ ساعة بعد نقع البذور. هذه الزيادة يتبعها زيادة أخرى تمتد إلى ما بعد ٧٢ ساعة من الإنبات ثم تتناقص تدريجياً بعد ذلك. أوضحت الدراسة انه بينما نشاط الإنزيمات التي تعمل على الكازين ترتبط معنوياً مع نسبة تكسير الجلوبيلين أثناء الإنبات، نجد أن إنزيم الأندوبيتيديز الذي يعمل على الأذوكول وإنزيم الهضم الذاتي مرتبطة ارتباطاً معنوياً مع بعض مكونات الجلوبيلين وغير مرتبطة مع البعض الأخر. بالإضافة إلى ما تقدم أظهرت بروتينات الهضم الذاتي والأندوبيتيديز والكازينولينك ارتباط معنوياً مع بعض نسبة تكسير الجلوبيلين في فترة الإنبات الممتدة بين ٤٨ إلى ٩٦ ساعة. من ناحية أخرى اظهر إنزيم الكربوكسي بيتيديز نشاط متزايد في المراحل الأولى من الإنبات، بينما اظهر إنزيم الأمينوبيتيديز نشاط ملحوظ في المراحل الأخيرة من الإنبات، كما اظهر إنزيم الأكسو بيتيديز ارتباط معنوياً مع كمية الأحماض الأمينية أثناء الإنبات. هذه النتائج أوضحت جليا دور الأكسو بيتيديز (إنزيم الكربوكسي بيتيديز وإنزيم الأمينوبيتيديز) في تكسير مكونات الجلوبيلين إثناء الإنبات، كما أوضحت أيضاً أن كلاً من الأكسوبيتيديز والأندوبيتيديز تعملان بطريقة متناغمة لتكسير الجلوبيلين في بذور الكتان إثناء الإنبات.

Key Words : Linseed storage proteins, Proteinases, Exopeptidases, Endopeptidases, Gel electrophoresis

ABSTRACT

The proteolytic activities of the germinating linseed using casein, azocoll and linseed globulin as substrates showed a steady increase until 48 hr of germination, followed with a rapid increase to reach a peak after 3 days lag of germination and then declined again. While caseinolytic activity is significantly correlated with the percent of degradation of the major globulin bands through out germination, endopeptidase measured with azocoll as substrate and autodigestive activities are significantly correlated with some bands and not with the others. Caseolytic, endopeptidase, autodigestive activities are

significantly correlated with the percent of degradation of the major globulin bands in the period of germination from 48 hr until 84 hr. The two exopeptidases investigated have shown different patterns. The carboxypeptidase was very active in the early period of germination, while aminopeptidase was active at the later stages. The activities of exopeptidases are significantly correlated with the amino acids content in the period of germination where the exopeptidase are at their maximum activities. This indicated their active role in the mobilization of the major globulin protein. In conclusion both the exopeptidase and endopeptidase work in harmony to regulate the degradation of the major globulin proteins of linseed.

INTRODUCTION

The storage proteins and some of the proteolytic enzymes are localized in the protein bodies [1-3]. During germination the protein bodies enlarge as a result of their increased osmotic potential due to proteolysis, the process that is found to be accompanied by a change in both the electrophoretic mobility and proteolytic activity [4-8].

Many authors [7-10] discussed the role of exopeptidase and endopeptidase in the mobilization of the storage reserves. They reported the major role of the endopeptidase in this process and the role of amino acids in the regulation of the proteases. However, the regulation process varies between the different genera [11].

In this study the activities of both the exopeptidase and endopeptidase in germinating linseed were investigated for the first time. These activities were statistically correlated with the percentage of degradation of the major globulin proteins and amino acids content of the germinating seeds to get a thorough conclusion on how storage protein utilization can be regulated in the linseed germinating seeds.

MATERIALS AND METHODS

A. GERMINATION STUDIES

Seeds of linseed (Linum usitatissimum l.) var Giza 5 (obtained from Agricultural Research Center, El-Giza, Egypt) were surface sterilized with 70% EtOH for 3 min. After rinsing thoroughly with distilled water, the seeds were transferred to 9-cm diameter petri dishes containing 6 ml distilled water per gram dry weight of the seeds. Germination was at room temperature (23 oC) in constant darkness. Seeds were harvested twice a day for 5 days, starting on the imbibition phase, during which time the cotyledons were carefully excised from all other portions of the seed. 15 grams of the cotyledons were lyophilized and then freeze-dried.

B. EXTRACTION

The meals of the freeze-dried cotyledons were extracted with 0.05 M borate buffer pH 8.0. The extract was clarified by centrifugation at 15000 rpm for 5 min. The recovered pellet was then re-extracted in the same manner for 5 min. Both extracts were combined.

C. ENZYME ASSAYS

The Leucineaminopeptidase and carboxypeptidase activities were assayed with L-leucine-p-nitroanilid (Leu-Nan) and α -N-benzyol-DL-arginine-p-nitroanilid (Bz-Arg-Nan) according the method of Siepen et al. [12]. Portions of the extract were assayed for caesin by the method of the Yemm and Cocking [13]. Enzyme activity was randomly expressed as the optical density of the released amino acids. Another portion of the extract was assayed for chymotrypsin activity by the method of Walsh and Wileox [14] using the synthetic substrate N-benzoyle-L-tyrosine ethylester (BTEE).

Endopeptidase was assayed using azocoll as a substrate. One ml of extract at pH 4.6 was incubated with 2 ml of H_2O and 2 ml of 0.1 N NaOH containing 2% Na_2CO3 were added, and the tubes were immediately centrifuged to remove the excess substrate [10]. The absorbency of the released dye was measured at 520 nm. Enzyme activity was randomly expressed as the optical density of the released amino acids.

D. AUTODIGESTIVE ACTIVITY

One ml of the extract was adjusted to pH 5.4, and then incubated at 35°C for 2 hr with 1 ml of At the end of incubation, proteins were precipitated with 1 ml of 15% TCA and removed by centrifugation. The amino acid content of the supernatant was determined using ninhydrin as a color reagent [13]. Enzyme activity was expressed in the same way as caseolytic activity.

E. GEL ELECTROPHORESIS

The seed meals were extracted with 0.125 M Tris/borate buffer, pH 8.9, containing 2% SDS and then analyzed on 12% PAGE following the method of Laemmli [15].

F. PROTEIN DETERMINATION

The protein content of linseed was determined by the method of Lowery et al. [16]

G. AMINO ACID DETERMINATION

The amino acid content of linseed was determined by the method of Lee and Takahashi [17].

H. STATISTICAL ANALYSIS

Regression analysis was computed by computer program STATSTICF.

RESULTS

A. DEGRADATION OF THE MAJOR STORAGE PROTEINS OF LINSEED DURING SEED GERMINATION.

Defatted meal of germinating linseed was extracted with 0.125 M Tris / borate buffer pH 8.9, containing 2 % SDS and analyzed on SDS-PAGE (Fig. 1A). The major globulin bands were gradually degraded on germination. The scans of the gel in Fig. 1A show a drop in the quantity of the major storage protein of linseed after 24 hr of germination (Fig. 1B). The major globulin bands show the same trend of degradation (Fig. 2). The intensity of the high molecular weight band (designated x) showed no change until 84 hr of germination (Fig. 1). Thereafter its intensity showed some change.

To confirm protein breakdown in the cotyledons during germination, changes in protein nitrogen and free amino acids nitrogen was measured (Fig. 3). Protein nitrogen in the cotyledons decreased rapidly during germination, while amino acids content remains nearly constant until 36 hr of germination and followed with an increase that coincides with the increase in some proteolytic activities.

B. PROTEOLYTIC ACTIVITY LEVELS DURING LINSEED GERMINATION.

0.05 M Tris/borate buffer pH 8 was used as an extractant for proteolytic assays in all proteolytic activities investigated except in case of endopeptidase against azocoll, 33 mM sodium acetate / acetic acid buffer pH 4.6 was used.

The level of chymotrypsin activity shows a slight increase in activity till 72 hr of germination and starts to decrease very slowly (Fig. 4). Regression equation between chymotrypsin activity and the percent of degradation of the major globulin bands gave negative non-significant correlation with r2 (regression coefficient square) = 0.008%. This value indicates that chymotrypsin activity had no role in the degradation of the major globulin.

As shown in Fig. 5A, caseolytic activity shows a steady increase in activity till 72 hr of germination. Thereafter the level of activity starts to fall sharply. The maximum activity is about 2.5-fold of that of dormant seeds. Regression analysis between the caseolytic activity and the percent of degradation of the major globulin bands gave high significant correlation with r2 from 59.75% to 83.63% (Fig. 5B). The values of r2 indicate that caseinolytic activity accounted for between 59.75% and 83.63% of the degradation of the major globulin proteins.

Endopeptidase activity measured with azocoll as a substrate followed nearly the same pattern of caseolytic activity (Fig. 6A). The results showed that regression coefficient squares between the percent of degradation of the major globulin bands and endopeptidase activity

account for between 37.24% and 53.54% of the degradation of the major globulin proteins. However while endopeptidase activity was significantly correlated with the percent of degradation of the major globulin band 2, it was non-significant with the other bands (Fig. 6B).

Autodigestive activity followed nearly the same pattern of caseolytic activity (Fig. 7A). The results also showed that the regression coefficient squares between the degradation percent of the major globulin bands and endopeptidase activity account for between 47.72% and 59.94% of the degradation of the major globulin proteins. While autodigestive activity was significantly correlated with the percent of degradation of the major globulin bands 2 and 3, it was non-significant with the other bands (Fig. 7B).

Positive significant correlation was recorded between the percent of degradation of linseed major bands, and caseolytic, endopeptidase and autodigestive activities in the period of germination from 48 hr to 84 hr (Fig. 5B, 6B, 7B).

The two exopeptidases investigated have shown different patterns; leucineaminopeptidase activity showed a steady increase until 36 hr of germination. Thereafter, the activity of leucineaminopeptidase showed a sharp increase and reached its maximum level (11-to 12-fold) after 48 hr of germination (Fig. 8). In case of carboxypeptidase, the activity curve shows two peaks, the first one is a wide peak with a plateau between 12 and 36 hr of germination (Fig. 8).

Regression analysis between leucineaminopeptidase or carboxypeptidase activity and percent of degradation of the major globulin bands through out germination gave positive non-significant correlation. However positive significant correlation was recorded between amino acids content and both leucineaminopeptidase and carboxypeptidase activity in the period of their maximum activities (Fig. 8B).

DISCUSSION

It was found that some of the linseed protein bands show high rate of degradation (band 1 and 2) and the others exhibit different rates. This pattern of breakdown is similar to that reported for the mung bean [18] and the pea [9] and different from that reported for *Phaseolus vulgaris* [6]. The degradation of the major globulin proteins was accompanied with an increase in proteolytic activity [3, 6, 8, 9].

Contrary to the work of Yomo and Srinivasan [19] and Yomo and Varner [20] on beans and peas respectively, who found that amino acids content decreases on germination, the present study clearly shows that the amino acids content increase slightly during germination. They also found that protease formation in attached cotyledons was inhibited by the presence of free amino acids, suggesting that level of the amino acids regulate the proteases in pea and beans. However this is not the case in linseed. These data support the suggestion that there may be more than one way for the seed to regulate proteolysis during the breakdown of the storage protein [11].

An outcome of the present investigation was the mechanism by which the seed regulated proteolysis during the breakdown of storage proteins, therefore the enzyme activities of both endopeptidase and exopeptidase were measured. Chymotrypsin activity showed no appreciable change during germination. However, the activity of chymotrypsin-like activity at the on-set of germination is higher than most of the proteolytic activities studied, indicating that the highest level of chymotrypsin-like activity which took place at the start of germination may be high enough to allow storage proteins breakdown [6]. However this suggestion can not be upheld because of the negative non-significant correlation between its activity and the percent of degradation of the major globulin proteins.

Although caseinolytic activity was significantly correlated with the percent of degradation of the major globulin proteins through out the germination process,

autodigestive and endopeptidase activities were significantly correlated with some bands and not with the others. However statistical analysis showed high significant correlation between caseolytic, endopeptidases and autodigestive activities, and percent of degradation of the major globulin protein in the period from 48 hr to 84 hr. These results suggest that endopeptidases are a multienzyme; some of them are active at the onset of germination, while the other act at the later stages.

The present data showed that carboxypeptiase activity (which released the carboxyl terminal amino acids from the polypeptide [11] increased during first two days of germination. This increment coincides with the degradation of the major bands into low molecular weight polypeptides. The great degradation of these polypeptides in the later stage of germination into dipeptides, dipeptidyl amides and tripeptides coincides with the accumulation of the leucineaminopeptidase activity which was reported [11] to hydrolyze the amino terminal amino acid residue from these metabolites. These data suggest that exopeptidase have a substantial role in the degradation of the major globulin proteins of linseed during germination.

The significant correlation between leucineaminopeptidase or carboxypeptidase and amino acids content during the time of their maximum activities supports the suggestion that exopeptidases have a substantial role in degradation of the major globulin proteins of linseed. However this finding contradicts early works which indicated that the exopeptidases play a minor role in degradation of the seed major storage proteins [3, 6, 8, 10]. However, the presence of some carboxypeptidases that persists the effect of PMSF inhibitor which inhibits to large extent the exopeptidases [10] ruled out this conclusion.

In conclusion both exopeptidase and endopeptidase work in hormony in mobilization of major storage proteins. Leucineaminopeptidase and carboxypeptidase act mutually to release the free amino acids required for the metabolism in early stages of germination

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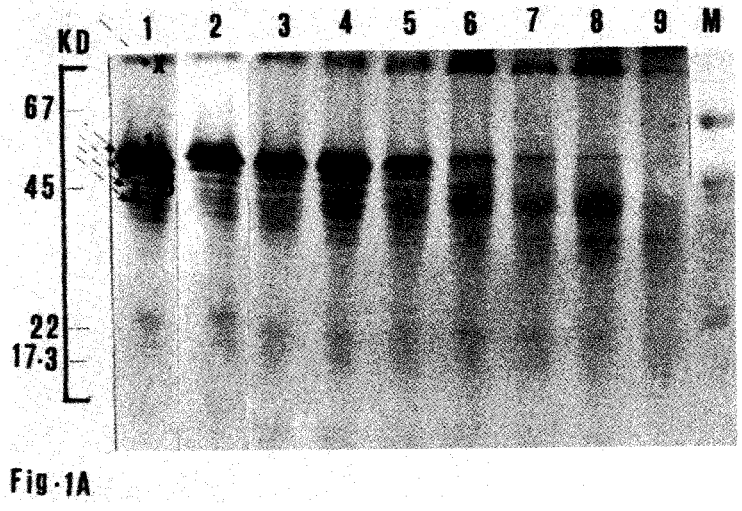

Fig·1A

Figure Legends

Fig. 1A. SDS electrophoresis patterns of germinating linseeds. Lane 1, mature linseed prior to germination; lane 2-9, after 12 to 96 hours of germination.

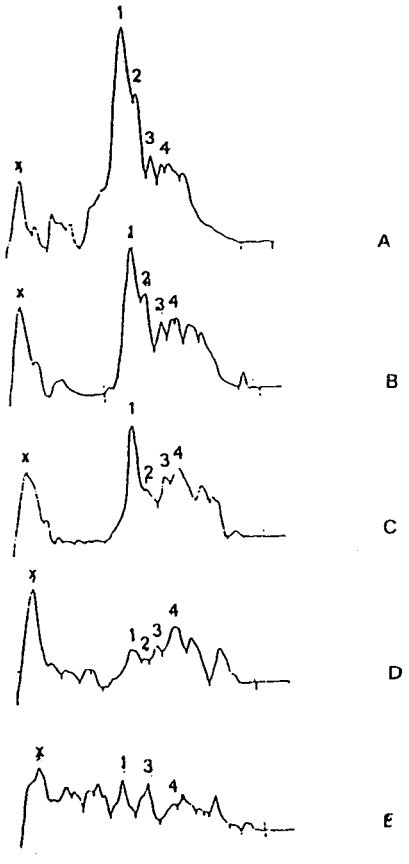

Fig. 1B. Scans of gel patterns of germinating linseed. A, mature seed prior to germination (zero time); B, after 24 h; C, after 48 h; D, after 72 h; E, after 96 h.

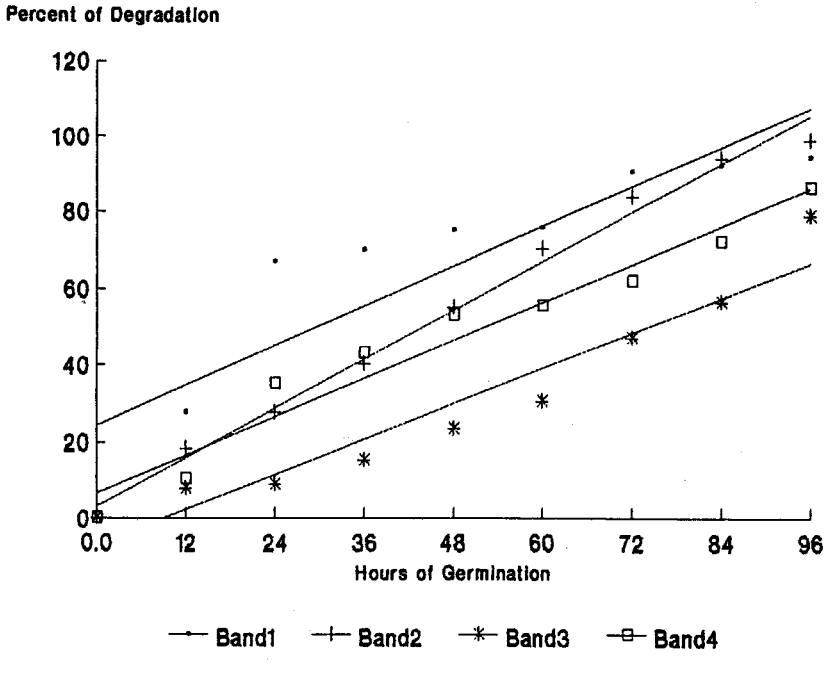

Fig. 2. Percent of degradation of the major globulin bands in germinating linseed.

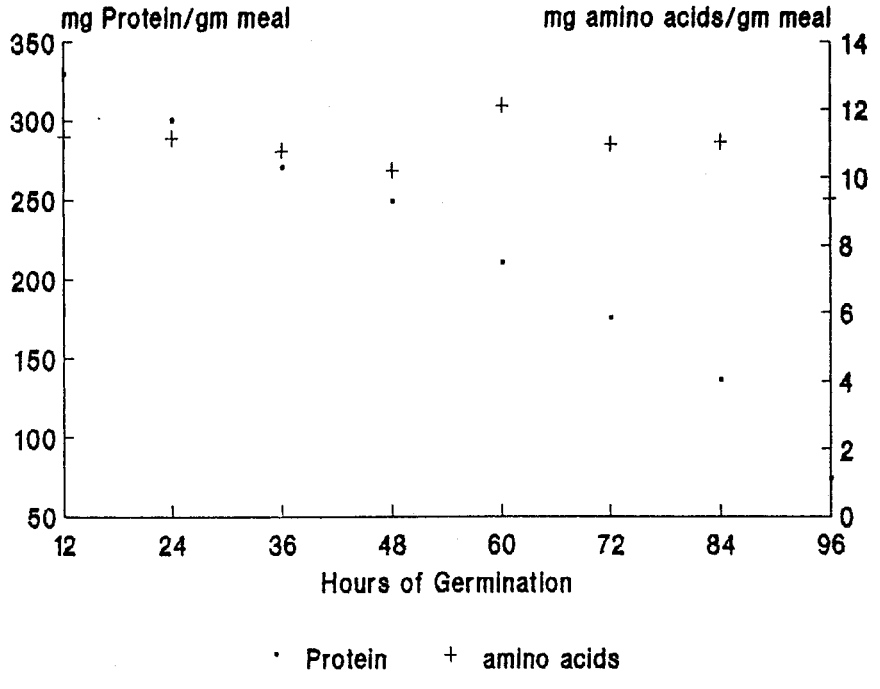

Fig. 3. Change in total proteins and amino acids contents in linseed during germination.

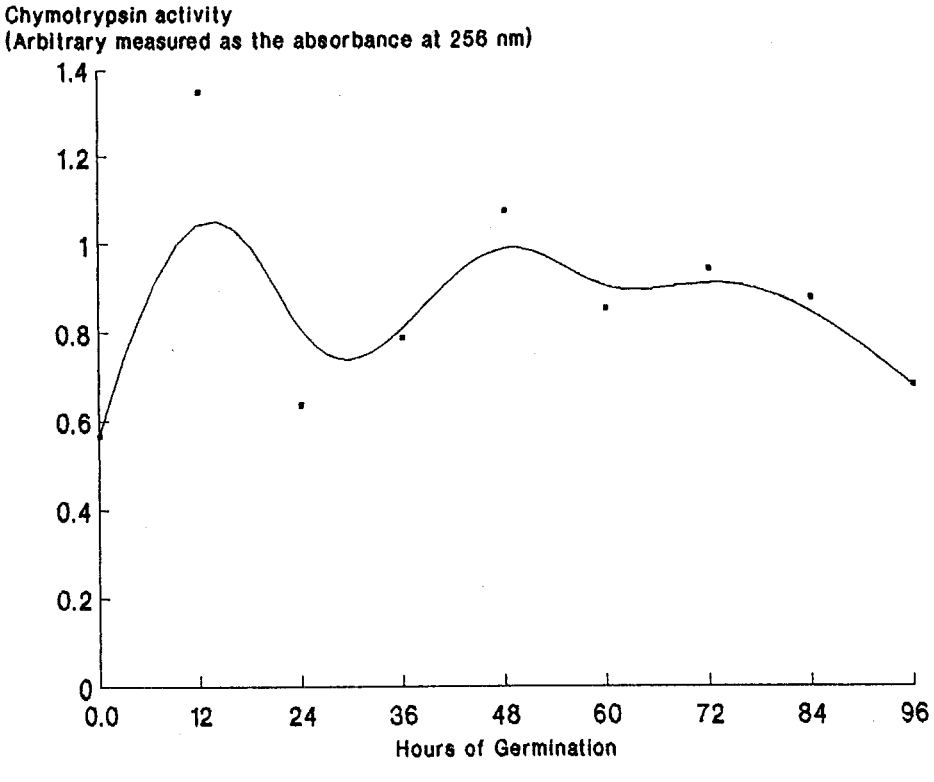

Fig. 4. Chymotrypsin activity during germination of linseed.

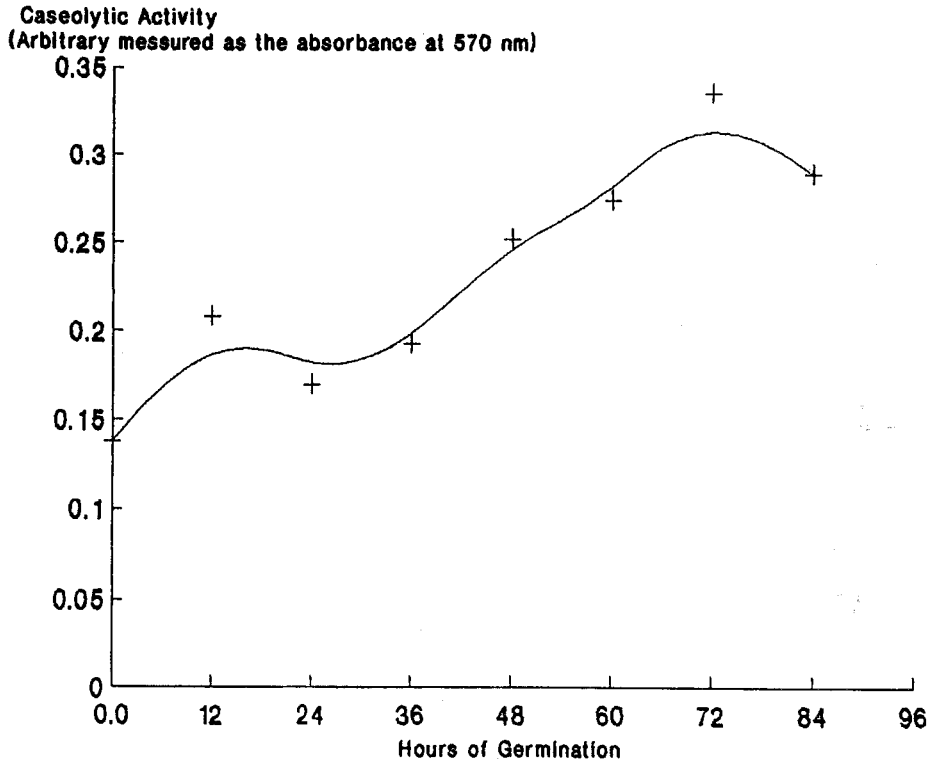

Fig. 5A. Caseolytic activity during germination of linseed.

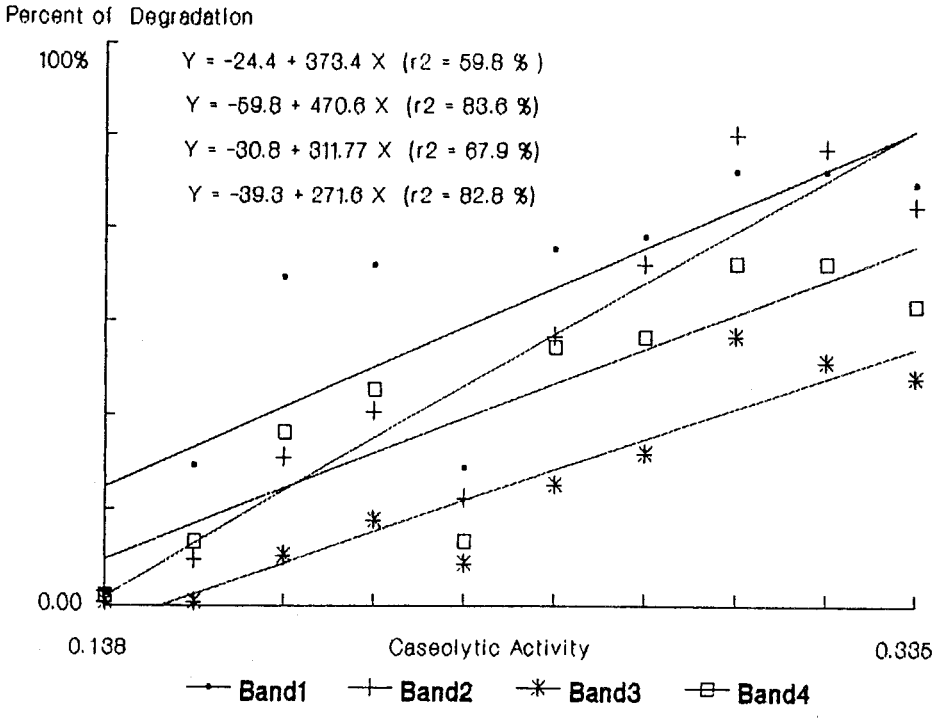

Fig. 5B. Scatter diagram showing the relationship between caseolytic activity and percent of degradation of the major globulin bands of linseed proteins, with regression lines equations.

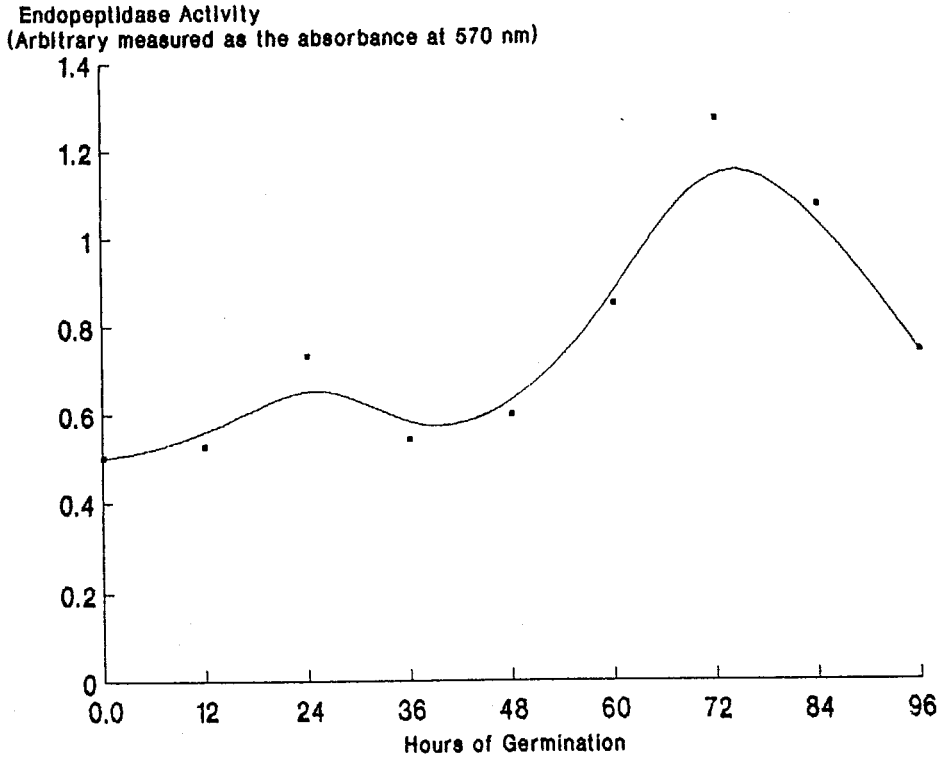

Fig. 6A. Endopeptidase activity during germination of linseed.

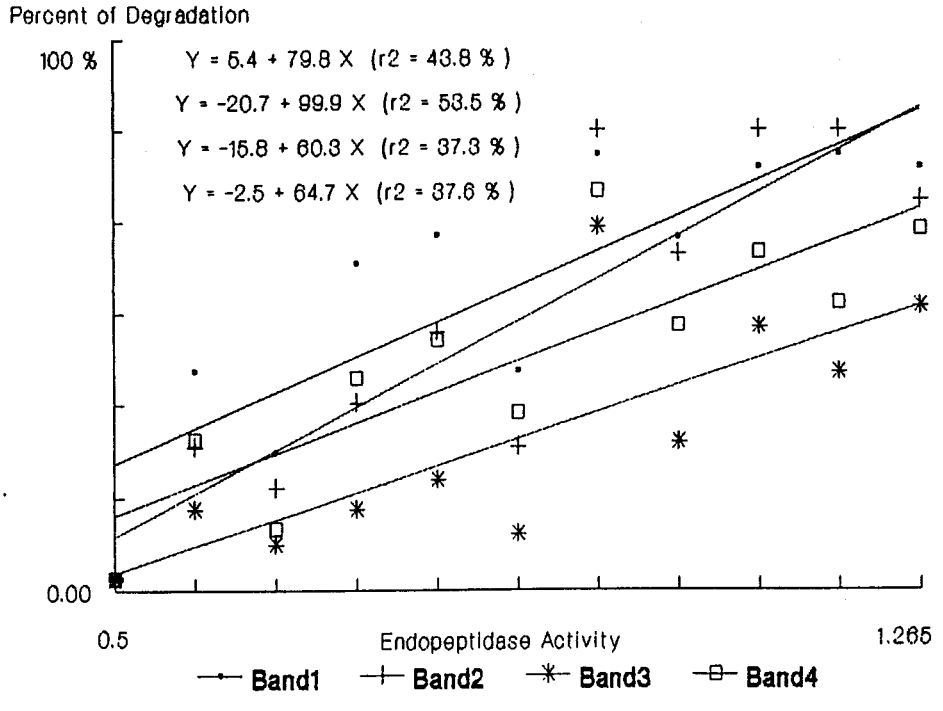

Fig. 6B. Scatter diagram showing the relationship between endopeptidase activity and percent of degradation of the major globulin bands of linseed proteins, with regression lines equations.

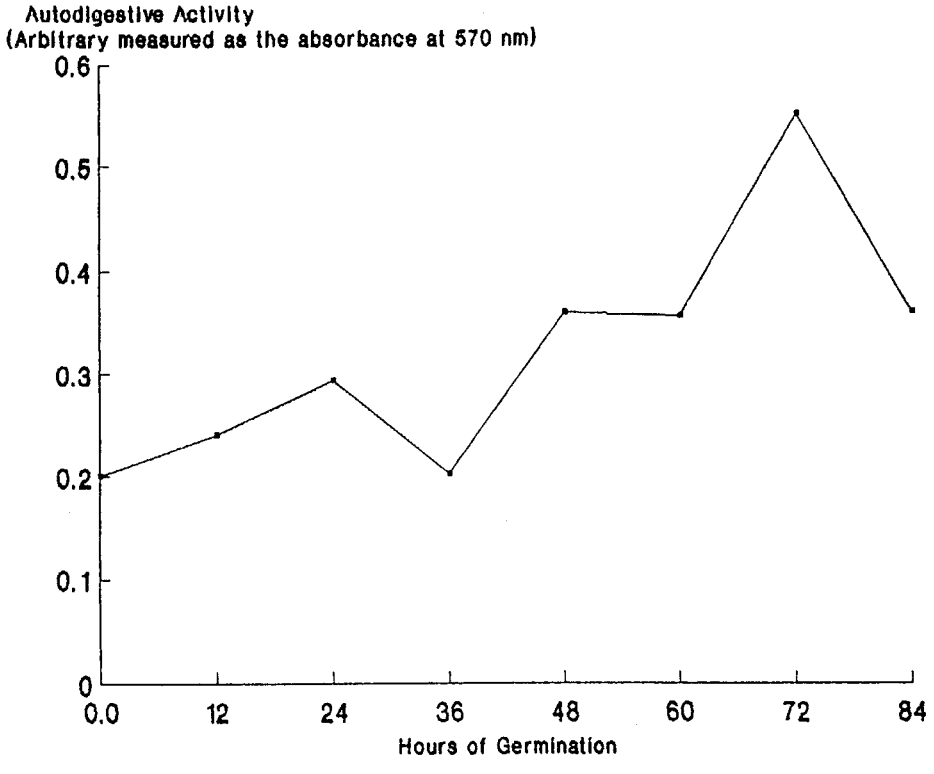

Fig. 7A. Autodigestive activity during germination of linseed.

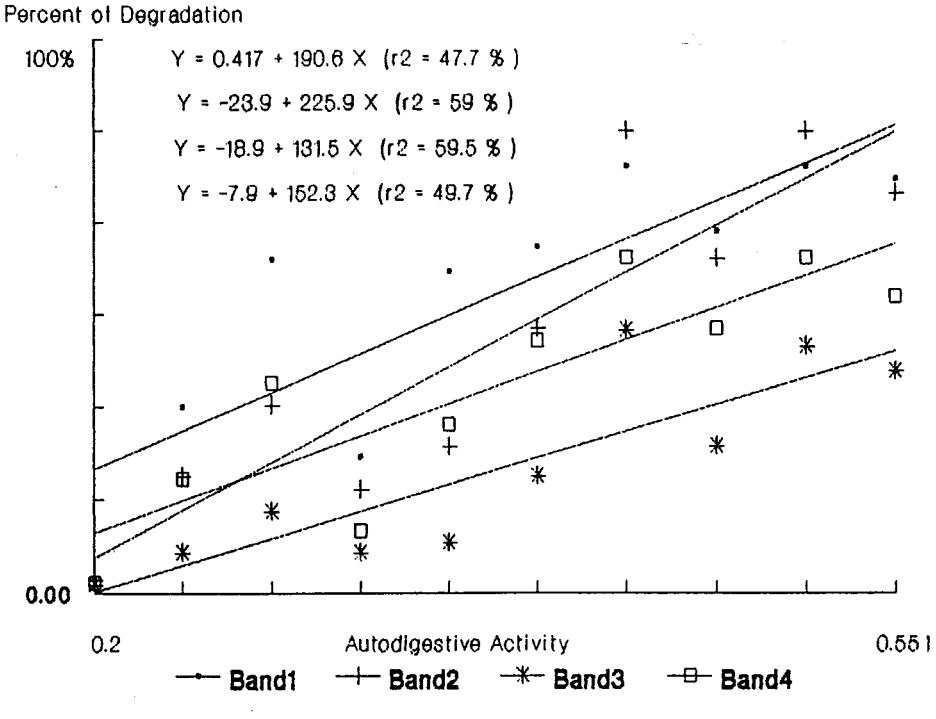

Fig. 7B. Scatter diagram showing the relationship between autodigestive activity and percent of degradation of the major globulin bands of linseed proteins, with regression lines equations.

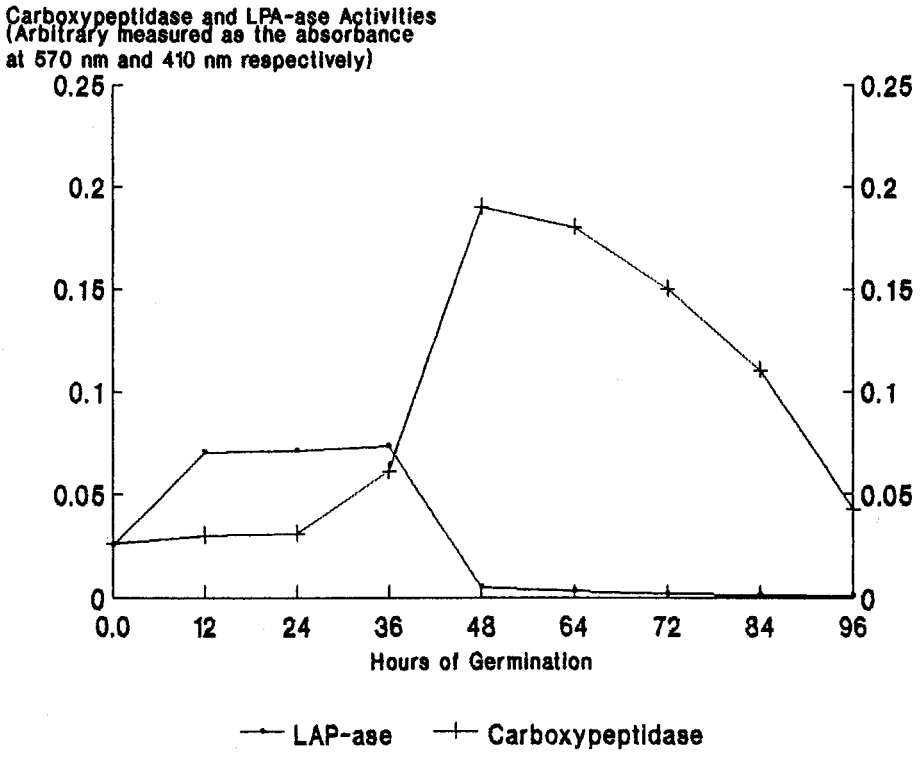

Fig. 8A. Leucineaminopeptidase activity during germination of linseed.

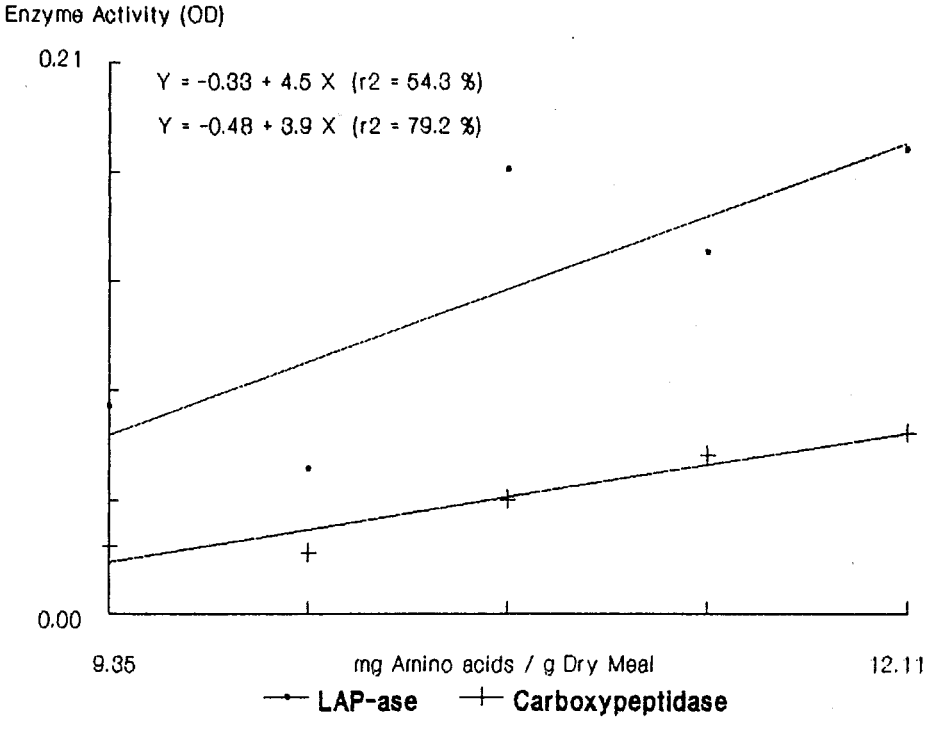

Fig. 8B. Scatter diagram showing the relationship between Leucineaminopeptidase activity and percent of degradation of the major globulin bands of linseed proteins, with regression lines equations.