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Protein Breakdown and Proteolytic Enzymes in Germinating Linseed

By

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With 8 Figures

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Summary

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The proteolytic activities of the germinating linseed using casein, azocoll and linseed globulin as substrats showed a steady increase until 48 h of germination, followed with a rapid increase reach a peak after 3 days lag of germination and then declined again. While caseolytic activity was significantly correlated with the percent of degradation of the major globulin bands throughout germination, endopeptidase measured with azocoll as substrate and autodigestive activities were 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 h until 96 h. 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 exopeptidases are at their maximum activities. This indicated their active role in the mobilization of the major globulin protein. In conclusion both exopeptidase and endopeptidase works in harmony to regulate the degradation of the major globulin proteins of linseed.

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Zusammenfassung

SAMMOUR R. H., EL-SHOURBAGY M. N., ABO-SHADY A. M. & ABASERY A. M. 1995. Proteinabbau und proteolytische Enzyme in keimenden Leinsamen. – Phyton (Horn, Austria) 35 (1): 25–36, 8 Abbildungen. – Englisch mit deutscher Zusammenfassung.

Die proteolytische Aktivität keimender Leinsamen wurde mit den Substraten Casein, Azocoll und Leinsamen-Globulin bestimmt. Nach ständiger Zunahme bis 48 Stunden Keimdauer erfolgt ein starker Anstieg mit einem Maximum nach 3 Tagen Keimung, danach sinkt die Aktivität wieder ab. Die caseolytische Aktivität korreliert während der Keimung signifikant mit dem prozentuellen Abbau der Hauptbanden von Globulin. Die Endopeptidase, welche mit Azocoll als Substrat gemessen wurde und die selbstabbauenden Aktivitäten sind signifikant mit manchen Banden korreliert, mit anderen wieder nicht. Die caseolytische Aktivität, die Aktivität der Endopeptidase sowie die selbstabbauenden Aktivitäten sind während der Zeit von 48 Stunden bis 96 Stunden Keimung signifikant mit dem Grad des Abbaues der Hauptbanden von Globulin korreliert. Die beiden Exopeptidasen zeigen ein unterschiedliches Verhalten. Die Carboxypeptidase ist sehr aktiv in den frühen Stadien der Keimung, während die Aminopeptidase zu einem späteren Zeitpunkt aktiv ist. Die Aktivitäten der Exopeptidasen sind mit dem Aminosäuregehalt während jener Phase der Keimung korreliert, in der die Exopeptidasen ihre maximale Aktivität besitzen. Dies weist auf ihre aktive Rolle bei der Mobilisierung des Hauptproteins hin. Zusammenfassend kann gesagt werden, daß sowohl Exopeptidase als auch Endopeptidase zusammenwirken, um den Abbau der Globuline im Leinsamen zu regulieren.

Introduction

The storage proteins and some of the proteolytic enzymes are localized in the protein bodies (SUNDBLOM & MIKOLA 1972, HOBDAY & al. 1973, MIKOLA 1983, CAPOCCHI 1988). 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 (KHAN & al. 1980, SMITH & al. 1982, NIELSEN & LIENER 1984, GALLESCHI & al. 1988, SAMMOUR 1989).

The role of exopeptidase and endopeptidase in the mobilization of the storage reserves was discussed by BASHA & BEEVERS 1975, CHRISPEELS & BOULTER 1975, SMITH & al. 1982, NIELSEN & LIENER 1984, GALLESCHI & al. 1988, SAMMOUR 1989. 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 was suggested to be varied between the different genera (RYAN 1973).

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.

Materials and Methods

1. Germination studies

Seeds of linseed *(Linum usitatissimum)*, 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 petri-dishes containing 6 ml distilled water per gram dry weight of the seeds. Germination was at room temperature (23 °C) in constant darkness. Seeds were harvested twice a day for 5 days, during which time the cotyledons were carefully excised from all other portions of the seed. A known weight of the cotyledons was lyophilized and then freeze-dried.

2. 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.

3. 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 & al. 1975. Portions of the extract were assayed for caeseolytic activity by the method of YEMM & COCKING 1955. Enzyme activity was randomely 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 & WILEOX 1970 using the synthetic substrate N-benzoyle-L-tyrosine ethylester (BTEE).

Endopeptidase was assayed using azocoll as a substrate. 1 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_2CO_3 were added, and the tubes were immediately centrifuged to remove the excess substrate (CHRISPEELS & BOULTER 1975). The absorbance of the released dye was measured at 520 nm. Enzyme activity was randomely expressed as the optical density of the released amino acids.

4. Autodigestive activity

1 ml of the extract was adjusted to pH 5.4, and then incubated at 35° C for 2 h with 1 ml of H_2O. 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 colour reagent (YEMM & COCKING 1955). Enzyme activity was expressed in the same way as caseolytic activity.

5. Gel electrophoresis

The seed meals were extracted with 0.125 M Tris/borate buffer, pH 8.9, containing 2 % SDS and then analysed on 12 % PAGE following the method of LAEMMLI 1970.

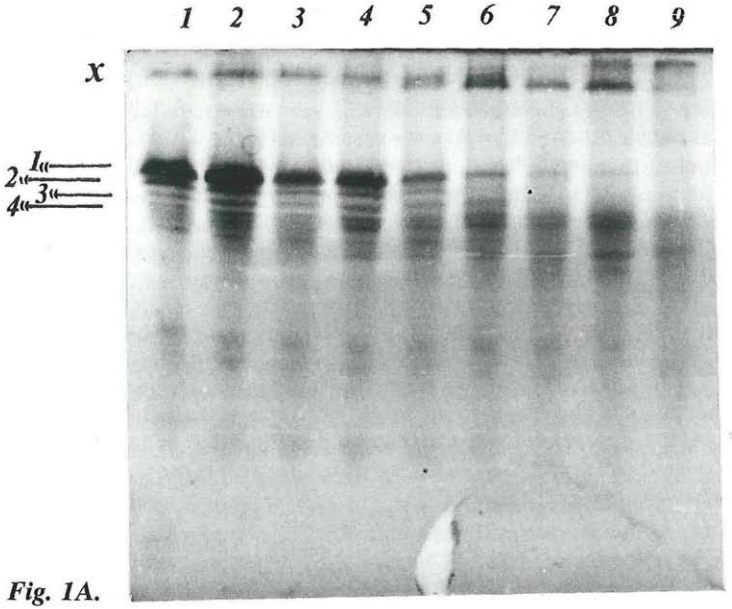

Fig. 1A.

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.

6. Protein determination

The protein content of linseed was determined by the method of LOWERY & al. 1951.

7. Amino acid determination

The amino acid content of linseed was determined by the method of LEE & TAKAHASHI 1966.

8. Statistical analysis

Regression analysis was computed by computer program STATSTICF.

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.

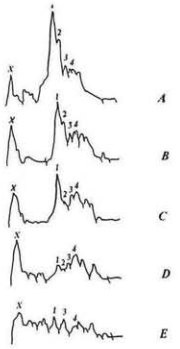

Fig. 1B

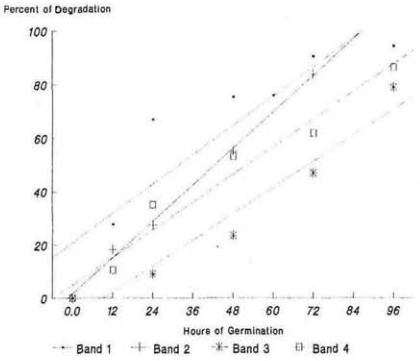

Fig. 2

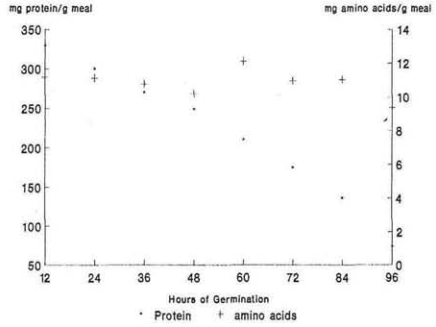

Fig. 3

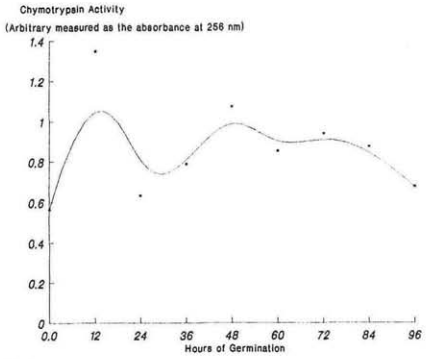

Fig. 4

Results

1. Degradation of the major storage protein of flax 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 drop in the quantity of the major storage protein of linseed after 24 h 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 h of germination (Fig. 1). After that time its intensity showed some change.

To confirm protein breakdown in the cotyledons during germination, changes in protein nitrogen and free amino acids nitrogen were measured (Fig. 3). Protein nitrogen in the cotyledons decreased rapidly during germination, while amino acids content shows slight decrease until 48 h of germination and followed with an increase that coincides with the increase in some proteolytic activities.

2. Proteolytic activity levels during flax seed 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 h of germination and start 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 correlations with r^2 (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 h of germination. After that time the level of activity starts to

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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.

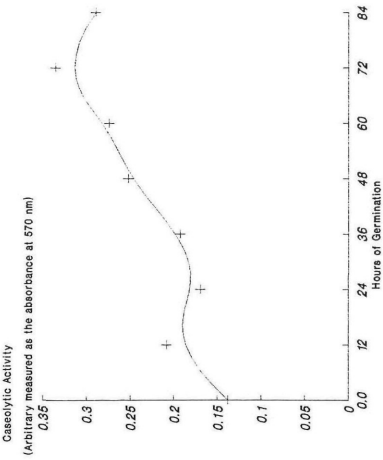

Fig. 5A

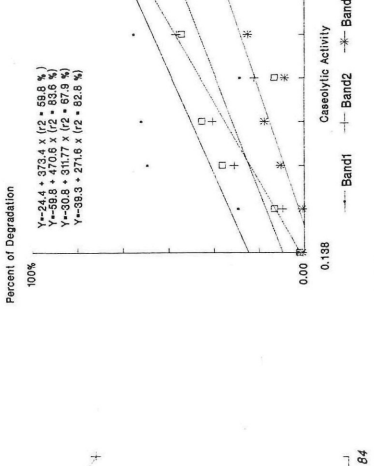

Fig. 6B

Fig. 6A

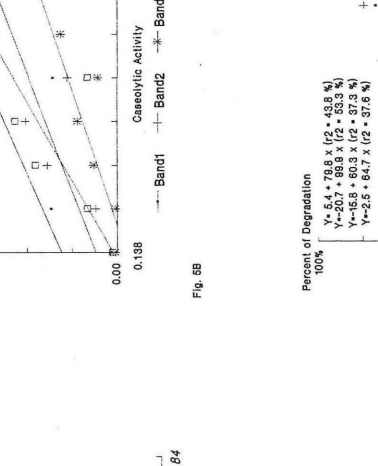

Fig. 6B

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 correlations with r^2 from 59.75 % to 83.63 % (Fig. 5B). The values of r^2 indicate that caseolytic 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 regression coefficient squares between the percent of degradation of the major globulin bands and endopeptidase activity indicate their account for between 37.24 % and 53.54 % of the degradation of the major globulin proteins. 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 regression coefficient squares between the degradation percent of the major globulin bands and endopeptidase activity indicate their 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 correlations were recorded between the percent of degradation of linseed major bands, and caseolytic , endopeptidase and autodigestive activities in the period of germination from 48 h to 84 h.

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

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 and carboxypeptidase activities during germination of linseed.

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

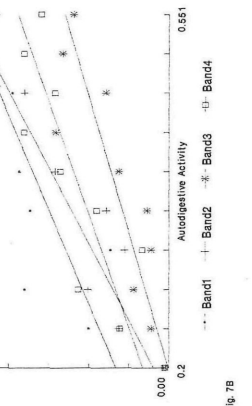

Fig. 7B

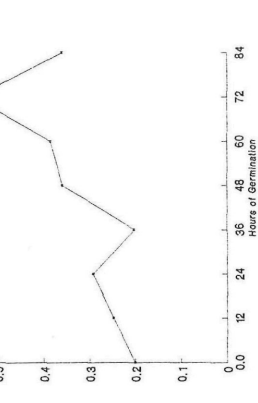

Fig. 7A

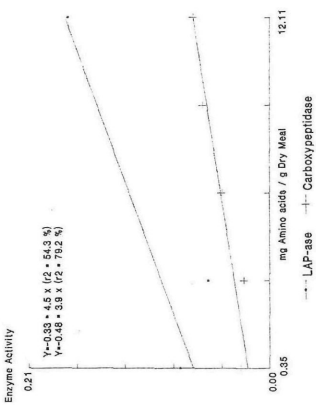

Fig. 8B

Fig. 8A

Regression analysis between leucineaminopeptidase or carboxypeptidase activity and percent of degradation of the major globulin bands throughout germination gave positive non-significant correlations. However positive significant correlations were 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 major globulin bands of linseed proteins 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 (BAUMGARTNER & CHRISPEELS 1976) and the pea (BASHA & BEEVERS 1975) and differs from that reported for *Phaseolus vulgaris* (NIELSEN & LIENER 1984). The degradation of the major globulin proteins was accompanied with an increase in proteolytic activity (BASHA & BEEVERS 1975, KHAN & al. 1980, SMITH & al. 1982, NIELSEN & LIENER 1984, SAMMOUR 1989).

The amino acids content showed slight increase during germination. These data contradict the work of YOMO & SRINIVASAN 1973, YOMO & VARNER 1973 on beans and peas who found that amino acids content decreases on 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 regulates 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 proteins (RYAN 1973).

Understanding the way the seed regulate proteolysis during the breakdown of storage proteins lead the author to measure the enzyme activities of both endopeptidase and exopeptidase.

Chymotrypsin activity showed no appreciable change during germination. However, the activity of chymotrypsin-like activity at the first set of germination is higher than most of the proteolytic activities studied, which lead to suggest that the highest level of chymotrypsin-like activity which took place at the start of germination may be high enough to allow storage protein breakdown (NIELSEN & LIENER 1984). However this suggestion can not be upheld because of the negative non-significant correlations between its activity and the percent of degradation of the major globulin proteins.

Although caseolytic activity was significantly correlated with the percent of degradation of the major globulin proteins throughout germination, autodigestive and endopeptidase activities were significantly correlated with some bands and not with the others. However statistical analysis showed high significant correlations between caseolytic, endopeptidase and

autodigestive activities, and percent of degradation of the major globulin protein in the period of germination from 48 h to 84 h. This leads to suggest that endopeptidases are a multienzyme ; some of them are active at the onset of germination, while the others act at the later stages.

The present data showed that carboxypeptidase activity (which releases the carboxyl terminal amino acids from the polypeptide (RYAN 1973) increased in the first two days of germination. This increase 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 the accumulation of the leucineaminopeptidase activity which was reported (RYAN 1973) to hydrolze the amino terminal amino acid residue from these metabolites. These data suggest that exopeptidases have a substantial role in the degradation of the major globulin proteins of linseed.

The significant correlation between leucine- aminopeptidase or carboxypeptidase and amino acids content during the time of their maximum activities supports the suggestion that exopeptidase activities have a substantial role in degradation of the major globulin proteins of linseed. However it has been reported earlier by many workers that exopeptidase play a minor role in such process, as germination was little inhibited when they subjected to inhibition with PMSF during germination (CHRISPEELS & BOULTER 1975, MIKOLA 1983, NIELSEN & LIENER 1984, SAMMOUR 1989). The presence of some carboxypeptidases which persist the effect of this inhibitor (CHRISPEELS & BOULTER 1975) ruled out this conclusion.

In conclusion both exopeptidase and endopeptidase work in harmony in mobilization of major storage proteins. Leucineaminopeptidase and carboxypeptidase exchange the function to release the free amino acids required for the metabolism during germination. It can also be concluded that endopeptidases are a multienzyme; some of them are active at the onset of germination, while the others act at the later stages.

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