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Changes in Glycinin-Digesting Protease Activity During Soybean Germination.

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Changes in glycinin-digesting protease activity during soybean germination have been investigated. The glycinin-digesting protease activities of imbibed or germinated soybean seed were assayed by RP-HPLC using a tryptic peptide from CM-glycinin or by SDS-PAGE using CM-glycinin as the endogenous substrate. Proteolytic activities of the germinated soybean seeds were found through the whole period of germination, the activities were maintained significantly unchanged during germination for 4 days, and then those specific activities declined slowly. AE-HPLC analysis of the glycinin-digesting protease in the imbibed or germinated soybean seeds showed unchanged peaks corresponding to glycinin-digesting activity, suggesting that the glycinin-digesting protease was not induced during germination but had already been synthesized during seed maturation.

Key words : Plant protease, glycinin, germination, *Glycine max*

Introduction

In our previous reports[1,2], we purified and characterized a glycinin-digesting serine protease from imbibed soybean seeds. The soybean serine protease with 98 kDa (98 kDa SBP) was revealed to hydrolyze the denatured glycinin $A_4A_5B_3$ complex at the neutral pH and one of the cleavage sites was identified to be carboxyl side of Tyr (378) of B_3 subunit. This fact that 98 kDa SBP can mobilize storage proteins such as glycinin $A_4A_5B_3$ complex suggest that this soybean protease can play a critical role in the early stages of germination However, it still remains obscure as to how these changes in the glycinin digesting protease activity take place during germination. In this report, we describe the changes in 98 kDa SBP activity and the breakdown of the storage proteins during germination.

Materials and Methods

Materials—Soybean cultiver Tamahomare, harvested at Okayama Prefecture, was used in this experiment. Glycinin complex was prepared from the soybean seeds by the method of Wolf et al.[3] and the carboxymethylated glycinin complex was prepared by the method of Crestfield et al.[4] The protein concentration was determined by the method of Lowry et al.[5] The peptidic substrate (Ala (375) -Asp-Phe-Tyr-Asn-Pro-Lys (381), PS-1) was purified from tryptic digest of CM-glycinin B3 subunit as described in our previous paper[2].

Enzyme assay—When a purified peptidic substrate was used, the proteolytic activity was expressed as the hydrolysis percentage of the peptidic substrate. The peptidic substrate (PS-1, 200 pmol) and soybean protease were incubated in 0.1 M ammonium bicarbonate, pH 8.2, at 37℃ for 2 hr. After termination of the reaction by boiling at 100℃ for 3 min, the remaining substrate and the fragments (Ala-Asp-Phe-Tyr) produced by the protease were analyzed and quantitated by RP-HPLC using Cosmosil 5C 18-AR column (Nalcalai Tesque, Inc. 0.45×25 cm) as described in our previous report[2].

Reverse Phase HPLC—The digested peptide in enzyme assay was analyzed by reverse phase HPLC on a JASCO 880-PU HPLC apparatus equipped with a Cosmosil 5C18-AR column (0.45×25 cm). The peptides were eluted with a linear gradient of 2-propanol from 0 to 70% in 0.1% TFA solution at a flow rate of 0.7 ml/min. Elution of peptides was monitored at 220 nm.

Electrophoresis—SDS-PAGE was done by the method of Laemmli[5] on 15% polyacrylamide gel in 0.05 M Tris-HCl buffer, pH 8.8, containing 0.1% SDS. The proteins were stained with 0.1% Coomassie Brilliant Blue R-250.

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Results and Discussion

Changes in specific activities of cm-glycinin digesting protease during soybean germination.

After 4h-imbibition in tap water, soybean seeds were placed on the wet cotton bed at 25℃ in the dark up to 7 days. Ten seeds in each incubation period were homogenized in cold acetone (100 ml) with a mortar and pestle. The homogenate was filtered by suction followed by air drying to powder. The defatted powder was suspended in 10 mM Tris-HCl buffer, pH 8.0 containing 2M NaCl for 4h. The extracts were filtered though four layers of gauze, and the filtrate were centrifuged at 10,000 g for 30 min. The resulting supernatants were taken to 50% ammonium sulfate saturation. After centrifugation, the precipitate was dissolved in a small amount of 20 mM Tris-HCl buffer, pH 7.0, containing 50 mM NaCl and dialyzed with the same buffer. The dialyzates were used as the crude enzymes. The specific activities of the crude enzymes prepared from the soybean seeds imbibed or germinated from 4h up 7 days were analyzed using the petidic substrate. As shown in Fig. 1, the activities continued unchanged for the first 4 days and then the specific activities gradually decreased. This result suggested that the glycinin digesting activity, which corresponds to 98 kDa SBP activity, is not induced during germination and is involved in the turnover of storage proteins, glycinin, in the early stages of germination.

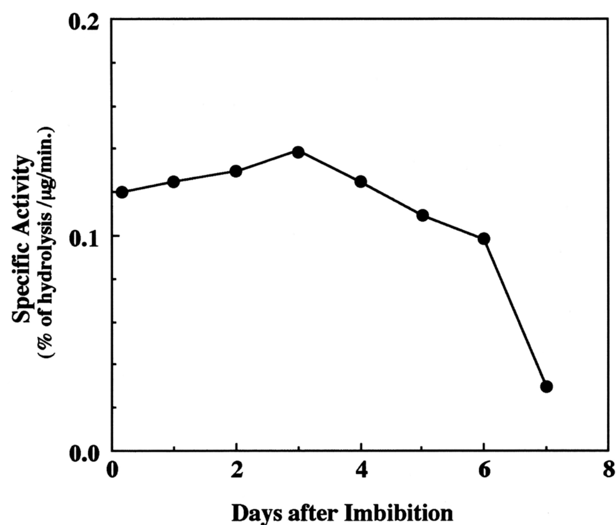

Fig. 1 Changes in specific activities of cm-glycinin digesting protease during soybean germination.
The cm-glycinin digesting activities were analyzed using a peptidic substrate from B3 subunit by RP-HPLC as described in Ref. 2.

Changes in expression of 98 kDa SBP during soybean germination

To confirm whether 98 kDa SBP is constantly expressed during seed germination, 98 kDa SBP activities were partially purified by anion exchange (AE)-HPLC from each crude enzyme fraction prepared above. Approximately 420 µg of crude proteins (220 µg for the sample of 7 days germination) was injected to a Jasco 880-PU HPLC apparatus equipped with a Jasco Intelligent UV/VIS detector (870UV) and a Shodex IEC QA-924 column (0.8×7.5 cm). The HPLC profiles of nine samples are shown in Fig. 2. The protein peaks indicated by arrows (around 18 min), which showed protease activity of 98 kDa SBP, remained constant through the whole period of germination. On the contrary, the broad protein peak around 25 min observed in seeds germinated from 0 to 2 days rapidly disappeared after 3 days incubation. Since the glycinin complex has been reported to consist of some acidic proteins and account for a large amount of storage protein of soybean, the broad protein peak appearing around

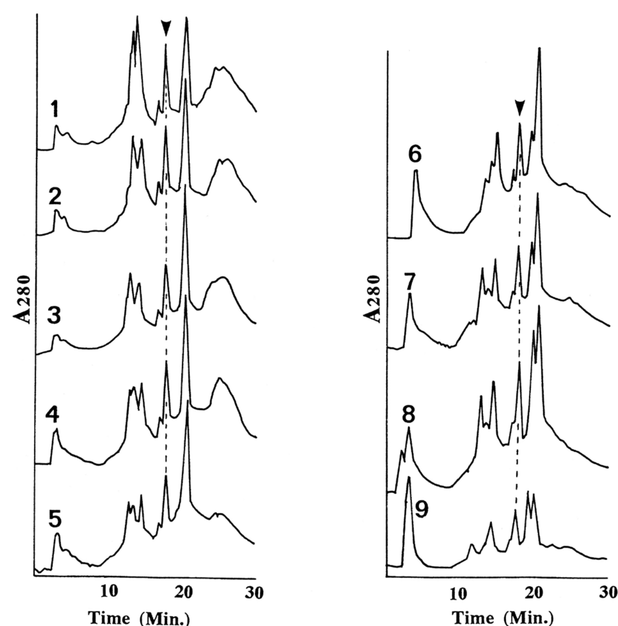

Fig. 2 AE-HPLC profiles of crude proteins prepared from imbibed or germinated soybean seeds.
Approximately 420 µg of crude proteases (220 µg for the sample of 7 days-germinated seeds) were used for HPLC analysis. 1, dry seeds ; 2, 4h-imbibed seeds ; 3, day 1 sample ; 4, day 2 sample ; 5, day 3 sample ; 6, day 4 sample ; 7, day 5 sample ; 8, day 6 sample ; 9, day 7 sample. Arrows indicate the elution position of 98 kDa SBP.

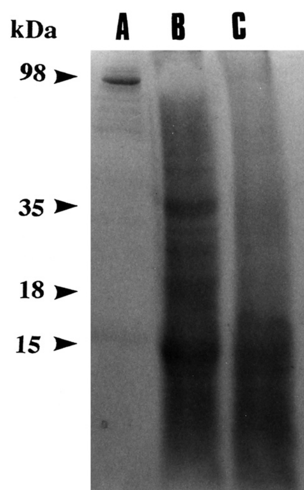

Fig. 3 SDS-PAGE of 98 kDa SBP purified from the extract of 4h-imbibed seeds by AE-HPLC in Fig. 2 and cm-glycinin incubated with 98 kDa SBP.
Lane A, 98 kDa SBP partially purified by AE-HPLC ; Lane B, cm-glycinin incubated with inactivated 98 kDa SBP in Lane A ; Lane C, cm-glycinin incubated with 98 kDa SBP in Lane A. 35 kDa, molecular mass of A4/A5 subunit ; 18 kDa, molecular mass of B3 subunit.

25 min were probably glycinin complex and hydrolyzed by 98 kDa SBP and/or another kind of glycinin digesting protease.

Purities of cm-glycinin digesting protease obtained by anion exchange (AE)-HPLC and cm-glycinin-digesting activity

The purities and the CM-glycinin digesting activities of 98 kDa SBP peaks were evaluated by SDS-PAGE by the method described in our previous report[2]. The purity and CM-glycinin digesting activity of the partially purified enzyme from 4h-imbibed seeds (Fig. 2-2) are shown in Fig. 3 as a typical example. 98 kDa SBP fractionated by AE-HPLC was found to be almost homologous and it completely hydrolyzed 35 kDa protein (A_4A_5 subunit)[1] and 18 kDa protein (B_3 subunit) in the glycinin complex. These proteolytic activities found in the other eight samples showed the same proteolytic property as that of purified 98 kDa SBP in our previous reports[2].

Conclusion

As a conclusion, it was found that 98 kDa SBP purified from the imbibed soybean seeds in our previous report[2] was not induced nor newly synthesized during imbibition or germination but had already been synthesized during seed maturation, and this glycinin-digesting protease is probably connected with the onset of the breakdown of storage proteins in response to seed imbibition.

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大豆発芽時期におけるグリシニン分解酵素の活性変動

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大豆発芽期におけるグリシニン分解酵素（98 kDa SBP）の活性変動を解析した．大豆種子を4時間水で膨潤後，25℃ 暗黒下で発芽させた．経時的にサンプリングを行い，2M NaCl を含むトリス緩衝液（pH 7.0）により粗酵素を抽出後，グリシニン由来のトリプシン分解ペプチドを基質としてグリシニン分解酵素の活性変動を逆相 HPLC により追跡した．その結果，種子膨潤後4日間比活性はほぼ一定の値を保ち，以後徐々に低下することが分かった．次いで，粗酵素溶液からイオン交換 HPLC により98 kDa SBP を部分精製するとともに，発芽期における 98 kDa SBP の消長を解析したところ，98 kDa SBP は乾燥種子及び各発芽段階の種子中全てに認められ，かつグリシン分解活性もグリシニン由来のトリプシン分解ペプチド基質に対する活性と同様に認められた．以上の結果から，98 kDa SBP は種子発芽に伴い誘導されるプロテアーゼではなく，種子貯蔵型のプロテアーゼであることが明らかになった．

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