Analysis of Adzuki Bean Seed Protein by Partial Amino acid Sequencing of Polypeptide Separated on Two-dimensional Gel

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Adzuki bean (*Vigna angularis* (WILLD.) OHWI et OHASHI) seed proteins were analyzed by two-dimensional polyacrylamide gel electrophoresis and the N-terminal or internal amino acid sequence determined. Several analyzed proteins were identified and mapped on two-dimensional polyacrylamide gel, as proteins homologous to legumin, vicilin and glycinin by homology search. All varieties which were analyzed in this experiment showed heterogeneities only in adzuki-vicilins on the electrophoresis profiles. Additionally, proteins whose N-terminal amino acid sequences were homologous to the internal amino acid sequences of pea vicilin were identified on 2D-PAGE profiles, and a deficient variety of these proteins was also identified in 15 varieties of adzuki bean.

KEY WORDS: amino acid sequence, post-translational proteolysis, seed protein, two-dimensional polyacrylamide gel electrophoresis, *Vigna angularis*.

Introduction

Seed proteins have been studied in several crops, for example legumins (CROY *et al.* 1982) and vicilin (LYCETT *et al.* 1983, HIRANO *et al.* 1983) in pea (*Pisum sativum* L.), glycinin (MOMMA *et al.* 1985) in soybean (*Glycine max* Merr.), and glutenin (FORDE *et al.* 1983) in wheat (*Triticum aestivum* L.). However, there have been no reports for adzuki bean (*Vigna angularis*) seed proteins, except a preliminary study of the amino acid sequence of trypsin inhibitor (KIYOHARA *et al.* 1981). Recently it has become possible to determine the N-terminal amino acid sequences of small quantities of proteins which have been separated by polyacrylamide gel electrophoresis (MATSUDAIRA 1988). For example, with the winged bean (*Psophocarpus tetragonolobus* L.), the seed proteins were characterized by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) pattern and the N-terminal amino acid sequence analysis (HIRANO 1989). In this report, characterization and identification of adzuki bean seed proteins were performed by 2D-PAGE analysis and the partial amino acid sequence analysis. Moreover, the compositions of adzuki bean seed proteins were compared by 2D-PAGE between 15 varieties and an adzuki-vicilin deficient variety, Momosesan, was identified.

Materials and Methods

Electrophoresis and electroblotting

Mature seeds of fifteen adzuki bean varieties (legend of Fig. 6) were ground to fine powder and proteins extracted using SDS sample buffer containing 10% (v/v) glycerol, 2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 62.5 mM Tris-HCl, and 0.1% (v/v) bromophenol blue for SDS-PAGE (LAEMMLI 1970) or lysis buffer containing 8 M urea, 2% (v/v) Nonidet P-40, 2% (v/v) ampholine (pH 3.5~11), 5% (v/v) 2-mercaptoethanol, and 5% (w/v) polyvinyl-

Received September 9, 1991.

Accepted August 26, 1992.

pyrrolidone for 2D-PAGE (O'FARRELL 1975). For sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) and the second dimension of 2D-PAGE electrophoresis, 15% (w/v) polyacrylamide gel was used. Electroblotting of proteins from polyacrylamide gel to polyvinylidene difluoride membrane was performed using a semi-dry blotting apparatus with a gradient buffer system (HIRANO 1989). The proteins on the polyacrylamide gel or the membrane were stained with Coomassie brilliant blue R-250 (HIRANO 1989) and the membrane subjected to the N-terminal amino acid sequence analysis.

Electroelution and partial proteolysis of protein in polyacrylamide gel

Proteins in 2D-PAGE gel (50 spots) were electroeluted from gel in electrophoresis buffer for 1.5 h with constant 1W (Electroelution apparatus, ISCO) and the electroeluted proteins were dialyzed against water. Proteins were dissolved in SDS sample buffer and 20 μ l of protein solution was applied to sample well of stacking gel. *Staphylococcus aureus* V8 proteinase (0.5 μ gml⁻¹ in 0.5 $\times$ SDS sample buffer) was overlaid on the protein solution. When the bromophenol blue dye reached the middle of stacking gel, the electrophoresis was halted for 30 min at room temperature (CLEVELAND *et al.* 1977). The separated polypeptides on the separation gel were electroblotted onto the membrane and subjected to the internal amino acid sequence analysis.

Amino acid sequence

Amino acid sequences were analyzed using protein sequencers (Applied Biosystems, 470A and 477A) (Matsudaira 1987). Homology search was performed using the DNASIS system (Takara Co.).

Results and Discussion

1. Analysis by electrophoresis

Proteins in several adzuki bean seeds were extracted with SDS sample buffer and separated by SDS-PAGE. Most proteins were detected at about 55 kDa as strongly stained bands (Fig. 1). These proteins were designated high-molecular-weight proteins (H), middle-molecular-weight proteins (M), and low-molecular-weight proteins (L). It was estimated by densitometry that band L, M, and H comprised 27.4%, 12.0%, and 12.4% (total 51.8%) of the total adzuki bean seed protein, respectively.

Proteins in adzuki bean seeds were

Fig. 1. SDS-PAGE of adzuki bean seed proteins. After the electrophoresis, proteins in polyacrylamide gel were stained by Coomassie brilliant blue R-250. Molecular weight markers consist of phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α -lactalbumin (14 kDa).

separated into several spots by 2D-PAGE (Fig. 2). The major bands at around 55 kDa which were detected by SDS-PAGE were separated into several spots with different molecular weights and isoelectric points. The isoelectric points of the major spots were almost identical and these spots overlapped each other in a 2D-PAGE profile. This overlapping of major spots in the 2D-PAGE pattern could be decreased if smaller amounts of sample were applied to the 2D-PAGE separation, though the other spots could not be detected (data not shown). Since they differed only in the isoelectric points, probably the difference was caused by minor substitutions or modifications of amino acids in the proteins.

2. N-Terminal amino acid sequence

Thirty-five prominent spots in the 2D-PAGE pattern stained by Coomassie brilliant blue were analyzed for the N-terminal amino acid sequences. The N-terminal amino acid sequences of thirteen out of the 35 analyzed were sequenceable (Fig. 3). Homology searching with a protein sequence data bank showed some proteins to be homologous to well-known proteins. Spot 1 had a homology with glycinin (MOMMA *et al.* 1985) in soybean and legumin (CROY *et al.* 1982) in pea. Seven amino acid residues in the sequence of Spot 1 were identical with those of legumin, and eight amino acid residues were also identical with those of glycinin. Spot 3 was similar to napin (CROUCH *et al.* 1983) in rape seeds. Though there were three amino acid deletions in the sequenced region, a protein which is homologous to wheat

Fig. 2. Electroblotted pattern of adzuki bean cv. Hayate shouzu seed proteins from 2D-PAGE gel. All spots were transferred from polyacrylamide gel to polyvinylidene difluoride membrane. Numbered spots were successfully determined the N-terminal amino acid sequence.

glutenin (Spot 6) (FORDE *et al.* 1983) was firstly observed in legume, and spot 12 also had a weak homology with glutenin. Spots 5, 7, 8, and 9, especially Spots 5 and 9, showed a high homology with the internal region of three kinds of vicilin which were vicilin type A (LYCETT *et al.* 1983), vicilin type B (LYCETT *et al.* 1983), and 14 kDa component of vicilin (HIRANO *et al.* 1982). Though these 4 spots were mapped at different positions on the 2D-PAGE gel, they showed a relatively high homology to each other. From these results, adzuki bean seeds were considered to contain both 7S globulin and 11S globulin, since legumin, glycinin, and napin are included in the family of 11S globulin and vicilin was in the family of 7S globulin.

3. Why some proteins could not be sequenced?

Adzuki bean trypsin inhibitor was known to be blocked at the N-terminal amino acid (KIYOHARA *et al.* 1981). Though nearly half of the spots examined was successfully analyzed in this experiment, the N-terminal amino acid sequence of the remaining half could not be sequenced. Nevertheless there was a sufficient quantity of proteins for sequencing. The reason why these proteins were not sequenced is probably that they had a blocking group at the N-terminus. We did not investigate which blocking group is involved in the blockage of adzuki bean seed proteins. Most of the blocked proteins in adzuki bean seed proteins were

Legumin	32	G	L	E	E	T	V	C	T	A	K	L	R	L	N	I	G
Legumin	333	G	L	E	E	T	V	C	T	A	K	L	R	L	N	I	G
1		G	L	D	E	T	I	G	H	H	W	L	Q	F	N	I	A
Glycinin	341	G	I	D	E	T	I	C	T	M	R	L	R	Q	N	I	G
2		I M V V E D D D E															
3		K	L	Q	E	E	Q	Q	E	S	A	F	V	Q	X	Y	R
Napin	45	K	Q	G	P	Q	Q	R	P	P	L	H	Q	Q	Y	C	N
4		H	F	E	Y	K	P	F	G	A	H	W					
13		H	L	E	I	Y	A	I	A								
12		I	V	X	X	E	X	Q	Q								
6		I	V	H	R	E	F	Q	Q	G	Q	Q	E	W	N	W	L
Glutenin						E	K	L	G	Q	G	Q	Q	P	R	Q	W
Vicilin (14.5)	31	S	R	R	S	V	S	S	E	S	G	P	F	N	L	R	S
Vicilin (A)	231	S	K	K	S	V	S	S	E	S	G	P	F	N	L	R	S
Vicilin (B)	55	S	K	K	S	V	S	S	R	S	E	P	F	N	L	K	S
5		H	K	K	A	L	H	H	Q	N	E	P	F	N	L	R	N
9		H	K	K	A	L	H	H	Q	N	E	P	F	N	L	R	N
7		H	K	K	A	L	H	H	Q	N	E	P	F	N	L	R	N
8		H	K	K	A	L	H	H	Q	N	E	P	F	N	L	R	N
10		I V A R E A A E S X E N V Y E F D G K N X P F Y X L H															
11		G S X X T N P F N I X Q L X A H															

Fig. 3. Results of N-terminal amino acid sequence analysis of adzuki bean seed proteins. Homologous proteins were compared and identical amino acids were indicated by solid-line boxes.

glycoproteins stained by the concanavalin A-peroxidase method (KIJIMOTO-OCHIAI *et al.* 1985) (data not shown). The major proteins (not spots 10, 11, 12, and 13 in Fig. 2) with about 55 kDa (Fig. 1) that were blocked at the N-terminus amino acid were also glycoprotein. These results suggest a relationship between glycosylation and the N-terminal amino acid blockage. Similar results concerning the N-terminal blockage and glycosylation were also reported for proteins in winged bean seeds (HIRANO 1989).

4. Internal amino acid sequence of major 55 kDa proteins

Prominent proteins with about 55 kDa in adzuki bean which showed heterogeneities were blocked at N-terminus by unknown modification (Fig. 2). Internal amino acid sequences of these proteins were analyzed by the method of limited proteolysis by *S. aureus* V8 proteinase (CLEVELAND *et al.* 1977) in polyacrylamide gel. The band L (Spot 12 in Fig. 2), which does not contaminate with band H and M in the major proteins with around 55 kDa, was electroeluted from gel and the internal amino acid sequence was determined using the limited proteolysis. The cleavage pattern of the proteolyzed band L by *S. aureus* V8 proteinase is shown in Figure 4. One of the bands obtained (arrowhead band) showed the internal amino acid sequence (Fig. 5). For others, it was possible to determine the amino acid sequence. This polypeptide showed homology (8 amino acids in 12 identified amino acids were identical) with vicilin (LYCETT *et al.* 1983) of pea. Vicilin is a 50 kDa protein; however the band L was estimated to have a molecular weight of 55 kDa by SDS-PAGE analysis. This difference of molecular weight could be caused by the addition of oligosaccharide as well as the mature glycosylated vicilin (GATEHOUSE *et al.* 1982), because vicilin homologous proteins in adzuki bean were also glycoproteins, which reacted with concanavalin A (data not shown). Otherwise some addition of amino acids to the protein was made during the evolutionary process. Major proteins were named adzuki-vicilin, because they were the major proteins in adzuki bean and a vicilin homologous protein.

5. Possibility of post-translational proteolysis of adzuki-vicilin

Vicilin of pea has been shown to be post-translationally proteolyzed into 19 kDa α -polypeptide, 13 kDa β -polypeptide, 12 kDa

Fig. 4. Limited proteolysis pattern of adzuki-vicilin by *S. aureus* V8 proteinase. The electroeluted proteins were digested in stacking gel for 30 min and proteolyzed peptides were tried, in order to analyze the amino acid sequence after the electroblotting from polyacrylamide gel to polyvinylidene difluoride membrane by protein sequencer.

Vicilin (A)	⁵⁹ V	<div style="border: 1px solid black; padding: 2px;">S</div>	S	R	S	<div style="border: 1px solid black; padding: 2px;">E P F N L</div>	K	<div style="border: 1px solid black; padding: 2px;">S</div>
Adzuki-vicilin	L	<div style="border: 1px solid black; padding: 2px;">S</div>	E	<div style="border: 1px solid black; padding: 2px;">E</div>	D	<div style="border: 1px solid black; padding: 2px;">E P F N L</div>	N	<div style="border: 1px solid black; padding: 2px;">S</div>
Vicilin (B)	²³⁴ V	<div style="border: 1px solid black; padding: 2px;">S</div>	S	<div style="border: 1px solid black; padding: 2px;">E</div>	S	G	<div style="border: 1px solid black; padding: 2px;">P F N L</div>	L
		<div style="border: 1px solid black; padding: 2px;">S</div>						<div style="border: 1px solid black; padding: 2px;">S</div>

Fig. 5. Internal amino acid sequence of adzuki-vicilin proteolyzed by *S. aureus* V8 proteinase and pea vicilin. Identical amino acids were indicated by solid-line boxes.

γ -polypeptide, 16 kDa γ -polypeptide, 33 kDa $\alpha + \beta$ -polypeptide (GATEHOUSE *et al.* 1982). Spots 5, 7, 8, and 9 in adzuki bean were homologous with 33 kDa vicilin of pea. Therefore, they might be a proteolyzed vicilin like the pea vicilin because they have decreased molecular weights, and showed sequence homology with the internal region of pea vicilins. The post-translational cleavage of adzuki-vicilin results in the absence of blockage of the N-terminal amino acid in spots 5, 7, 8, and 9. Though the proteolyzed subunits of vicilin were efficiently observed in pea seed, the proteolyzed adzuki-vicilin were not efficient as in the case of pea. This observation suggests that proteolysis of vicilin in adzuki bean like peas was less efficient than that in pea.

Vicilins in other species did not cleave post-translationally (PERNOLLET and MOSSE, 1983). However, we have neither observed the post-translational proteolysis of vicilin-like protein by *in vitro* pulse chase experiments, nor identified the proteinase for the post-translational proteolysis. To prove the post-translational proteolysis in adzuki bean, preparation of a specific antibody against adzuki-vicilin is now under experiment.

6. Varietal difference was only observed for adzuki-vicilin

No significant varietal differences were detected by SDS-AGE analysis (data not shown) because of the overlapping of several bands. Fifteen varieties were compared for 2D-PAGE pattern, and only adzuki-vicilin showed heterogeneities between the varieties (Fig. 6). No. 1 to No. 10 patterns showed rich spots of the subunits of adzuki-vicilin in the relative basic isoelectric points region like Chunagon. However, No. 11 pattern to No. 15 patterns showed rich spots of the subunits of adzuki-vicilin in the relative acidic isoelectric points like Dainagon. The number of spots and the patterns of adzuki-vicilins were significantly different from each other. For example, Kantou-2 (No. 13) has 13 spots and Chuunagon (No. 1) has 17 spots of adzuki-vicilin. Momosesan (No. 3) did not have any Spots 5, 7, 8, and 9. If these polypeptides were produced by post-translational cleavage of adzuki-vicilin, this Momosesan might be a variety deficient in proteinase for post-translational proteolysis of adzuki-vicilin, or non-sensitive for the proteinase by a point mutation adzuki-vicilin at the cleavage site. Other than this, Momosesan lost the subunit which was post-translationally cleaved and the subunit which was not affected the post-translational proteolysis remained in seed. The origin of the Momosesan was completely different from the other varieties which were analyzed in this study. Momosesan would be an useful variety for the study of post-dtranslational proteolysis of adzuki-vicilins.

Finally, we note that the study of partial amino acid sequence analysis would contribute to molecular biology in the identification of protein by homology search. The information

Fig. 6. Varietal difference of adzuki-vicilins on 2D-PAGE profile. Fifteen kinds of varieties were analyzed; 1, Chunagon from Nagano; 2, Hatsune-shouzu from Hokkaido; 3, Momosesan from Shiojiri city, Nagano; 4, Hayate-shouzu from Hokkaido; 5, Ina-zairai from Ina city, Nagano; 6, Cha-adzuki from Okayama; 7, Hokkai-shiro-shouzu from Hokkaido; 8, Shouchouhin from Akeno Town, Nagano; 9, Aka-adzuki from Nagano; 10, Akane-dainagon from Hokkaido; 11, Beni-dainagon from Hokkaido; 12, Erimo-shouzu from Hokkaido; 13, Kantou-2 from Nagano; 14, Col/Tokushima/1967 from Tokushima; 15, Dainagon from Hokkaido.

from amino acid sequences would be helpful in predicting the function of the proteins, and for molecular cloning.

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2次元電気泳動によって分離したポリペプチド鎖アミノ酸配列によるアズキ種子タンパク質の分析

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アズキ種子タンパク質を2次元ポリアクリルアミドゲル電気泳動を用いて分離した。アズキ種子タンパク質はデンストメーターによる測定の結果, 55 kDa付近に存在するタンパク質が全種子タンパク質の50%以上を占めていることがわかった (Fig. 1)。ほとんどのアズキ種子タンパク質は中性付近に等電点を持つタンパク質であった (Fig. 2)。2次元ポリアクリルアミドゲル電気泳動で分離されたタンパク質を同定するために, 分離されたタンパク質をポリビニリデンジフルオリド膜にエレクトロブロットイングし, そのN末端アミノ酸配列の解析を試みた。幾つかのタンパク質はN末端アミノ酸が修飾された状態にあると考えられ, N末端アミノ酸配列を分析することは出来なかった。分析出来たタンパク質のアミノ酸配列は, これまでにアミノ酸配列が決定されているグルテニン, ナーピン, ビシリン, およびクリシニンの配列と高い相同性のあることがわかった。55 kDa付近の主要なタンパク質についてはN末端アミノ酸配列の解析が十分にできなかったので, *Staphylococcus aureus* V8プロテアーゼによって部分解析することにより, その内部アミノ酸配列 (Fig. 3) を調べたところ, ビシリンとの相同性が認められた (Fig. 4)。ビシリンと相同性を持つタンパク質が高分子量域 (55 kDa) と分子量域 (30 kDa) において同定されたので, アズキ種子内においてもエンドウの場合と同じように内生プロテアーゼによる分解が起きているのではないかと推察された。ただし, これは低分子量のビシリンと相同性のあるタンパク質が全く別な遺伝子から発現している可能性も考えられるので, 今後, 特異的な抗体などを用いて詳しい解析が必要であると思われる。アズキの主要な15品種において, その品種間差異を2次元ポリアクリルアミドゲル電気泳動によって調べたところ, アズキ種子タンパク質の変異は55 kDa付近のビシリンと相同性のあるタンパク質に集中 (Fig. 5) しており, スポットの数やそのタンパク質量において大きな変異が見られた (Fig. 6)。また, 調査した15品種中の1品種においては, 低分子量のビシリンと相同性のあるタンパク質を欠失しているものが観察された。この品種は低分子量のビシリンに相同性のあるタンパク質が, 内生プロテアーゼによる分解によるのか, 全く別の遺伝子に起源を持つのか調べるのに役立つと考えられた。