Note

Structural and Immunochemical Homologies between Foxtail Millet Glutelin 60 kDa and Starch Granule-bound Starch Synthase Proteins from Rice, Barley, Corn and Wheat Grains

Kenji TAKUMI,¹ Junko UDAKA,² Masumi KIMOTO,³ Tetsuzo KOGA⁴ and Hideaki TSUJI³

¹Department of Food and Nutrition, Okayama Women's College,
Kurashiki, Okayama 710–8511, Japan

²Laboratory of Food and Nutrition, Faculty of Education, Ehime University,
Matsuyama, Ehime 790–826, Japan

³Department of Nutrition, Faculty of Health and Welfare, Okayama Prefectural University,
Sojya, Okayama 719–1112, Japan

⁴Department of Nutrition, University of Tokushima,
Tokushima, Tokushima 770–0042, Japan

(Received October 14, 1999)

Summary Foxtail millet glutelin 60 kDa (MG60) was purified by preparative SDS-PAGE, and the N-terminal amino acid sequence was determined within 20 residues. The result demonstrated that the primary structure at N-terminal of MG60 was almost identical to those of the granule-bound starch synthase (GBSS) proteins from rice, barley, corn, wheat and potato. The existence of common epitopes among MG60 and GBSS proteins from these starch-storing cereals were corroborated by immunoblot analysis using antisera raised against MG60. These facts strongly suggest a close relationship between MG60-like glutelins and GBSS proteins.

Key Words foxtail millet, glutelin 60 kDa, N-terminal amino acid sequence, starch granule-bound starch synthase, antigenic homology.

Our previous paper (1) indicated that the prolamins from foxtail (Italian), proso (Common) and sawa (Japanese) millet cultivars contained common epitopes among them. Since then, however, little information on the physico- or immuno-chemical properties of the millet storage proteins has been published so far as we know. Miflin and Shewry (2) have proposed that glutelin proteins serve a metabolic and/or structural role rather than a storage function in some cereals. Recently, we purified a polypeptide a component with a molecular mass (MM) of 60 kDa (referred to as MG60) from the urea-soluble glutelin of a foxtail millet cultivar. Then, the N-terminal amino acid sequence of MG60 was determined by the method of Matsudaira (3) in order to obtain the first clue about the function of the newly obtained glutelin component.

In this paper, we describe the N-terminal amino acid sequence homology and antigenic similarity among urea-soluble MG60-like glutelins from rice and other starch-storing cereals (barley, corn and wheat). Foxtail millet (*Setaria italica* Beau, var *akiawa*) and corn were supplied by Dr. T. Ishii (Laboratory of Culture, Faculty of Education, Ehime University). Rice, barely, and wheat were purchased from Kaneko Seed Company (Maebashi Gunma, Japan). All the cereal seeds hulled and milled were passed through a 0.5 mm sieve, defatted with acetone and air-dried as described previously (1). The defatted flours (5 g) were then extracted twice with 1 M

NaCl (100 mL) and 70% ethanol (100 mL) with stirring for 1 h, followed by centrifugation at 21,000×*g* for 20 min at 20°C. After the supernatants were removed, the salt- and ethanol-insoluble residues were washed twice with water followed by washing with acetone three times as described previously (3). The dried residue was used for the extraction of glutelin fractions or components of the millet flour. The buffer used here contained 5 M urea, 2% SDS and 0.5% 2-mercaptoethanol in 50 mM Tris/HCl buffer (pH 8.6) (buffer A). The dried residue (2 g) was suspended in 50 mL of the solvent and stirred for 30 min at room temperature. After being centrifuged as stated above, the supernatants were pooled, to which an equal volume of acetone was added, and the resulting pellets were washed three times with acetone. (The content of glutelin fraction was 0.35 g in dry weight, corresponding to a recovery of 17.5%. Further fractionation and purification of glutelin were done by a preparative SDS-PAGE (4), and one major component with a MM of 60 kDa was obtained. The glutelin, MG60, was subjected to two-dimensional electrophoresis by the method of O'Farrell et al. (5). As shown in Fig. 1, MG60 was a basic and monomeric component. The N-terminal amino acid sequence of MG60 was determined by the method of Matsudaira (3). MG60 was electrophoresed on a SDS/15% polyacrylamide gel (1 mm in thick) and transferred from the gel to a polyvinylidene difluoride

(PVDF) membrane. Then, the protein band corresponding to MG60 was sequenced by automated Edman degradation with a Shimadzu PSQ-2 protein sequencer. The results thus obtained, together with data from Swiss prot protein database 34.0 and from other workers, are listed in Table 1. Although the sequence was determined within 20 residues, the primary structure of MG60 was found to be quite similar to that of granule-bound starch synthase (EC 2.4.1.11) in rice, and allied to those in barley, corn (maize) and wheat in homologies of 90, 90 and 75%, respectively. The granule-bound starch synthase (GBSS), called *waxy* protein, is a major granule-bound ADP transferase responsible for the synthesis of amylose and aminopectin (6) in starch-storing cereals and tubers. The MMs of GBSS range from 58 to 60 kDa. For example, Echt and Shwartz (7) first reported a nuclear-encoded enzyme of about 60 kDa, which is active in the starch-synthesizing plastids of plants (8, 9). Clark et al. (10) have clarified the

Fig. 1. Two-dimensional electrophoresis of the purified MG60 from buffer A-soluble glutelin. First electrophoresis was conducted in non-equilibrium pH gradient electrofocusing (NEPHGE) containing 5% ampholine (pH 3.5–10). Subsequently, SDS-PAGE was done with a 15% polyacrylamide gel. The right number represent MMs of marker proteins: phosphorylase (94 kDa), bovine (serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor 20 kDa), α -lactalbumin (14.4 kDa).

complete nucleotide sequence for the *waxy* protein of hexaploid wheat and suggested that the mature *waxy* proteins of all species investigated are 58–60 kDa, as is the case with wheat. Sano and Hirano (11, 12) verified that the mature *waxy* gene product from corn, and that from potato, was done as 60 kDa (13, 14). These data strongly-suggest that the starch-storing cereals or tubers share the buffer A-soluble glutelin components. As for the immunochemical relationship between MG60 and GBSS, this was examined by immunoblot analysis (15) using rabbit anti-MG60 antiserum that was prepared by a procedure described previously (16). At first, the antigenic specificity of the antiserum to anti-MG60 was determined against several millet proteins as shown in Fig. 2. Of these protein fractions, only the buffer A-soluble glutelin displayed clearly a positive reaction with the anti-MG60 antiserum, and few cross-reactions were observed for the prolamin fraction (lane 2) as well as the albumin and globulin fractions (lane 1), proving the high antigenic specificity of the antiserum. To confirm the existence of MG60-like glutelin in rice and other starch-storing cereals, the buffer A-soluble glutelin fractions were prepared as described above and subjected to immunoblot analysis. Figure 3 reveals that the individual buffer A-soluble gultelin fractions contain only one polypeptide component with an apparent MM of nearly 58–60 kDa in rice (lane 1), barley (lane 2), corn (lane 3) and wheat (lane 4), but not in rye (lane 5) or oat (lane 6). These results indicated that buffer A-soluble glutelin components from the starch-storing cereals shared MG60-like glutelin poly-peptides. In rye and oat, however, the buffer A-soluble glutelin fractions failed to show any antigenic responses. Concerning this, we have no idea except that their starch enzymes were not a granule-bound type but a soluble one, or that the cultivars used in this study were accidentally *waxy* mutants which are defects or scare in the bound starch enzyme (17). With oat, its storage proteins are saline-soluble globulins but not glutelin (18).

In any case, globulin may well be useful for the detec-

Table 1. Comparison of N-terminal amino acid sequence between MG60 and its likeness from rice and other starch-storing cereals.

Source	Sequence																				Homology (%)	
	1				5				10				15				20					
MG60 ^a	A	T	G	A	G	M	N	V	V	F	V	G	A	E	M	A	P	W	S	K		
Rice ^{b,c,d}	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	20/20 (100)
Barley ^{c,d}	*	*	*	*	S	*	*	*	L	*	*	*	*	*	*	*	*	*	*	*	*	18/20 (90)
Maize ^{c,d}	*	*	S	—	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	18/20 (90)
Wheat ^e	T	G	S	G	*	*	*	L	*	*	*	*	*	*	*	*	*	*	*	*	*	15/20 (75)
Potato [†]	—	—	*	K	*	*	*	L	I	*	*	*	T	*	V	*	G	*	*	*	*	12/20 (60)

^a Foxtail millet glutelin 60 kDa subunit (in this paper).
^b Swiss-prot protein sequence database release 34.0 (in this paper).
^c Van der Leij et al. (Fig. 2 in ref. 14).
^d Hirano et al. (Fig. 5 in ref. 12).
^e Clark et al. (Fig. 1 in ref. 10).
[†] Potato is cited as an example for starch storage tubars.

Fig. 2. Immunoblot analyses of foxtail millet protein fraction using anti-MG60. Panel A: SDS-PAGE profiles of the millet proteins. Lanes: 1, salt-soluble fraction sharing albumin and globulin; 2, ethanol-soluble prolamins; 3, buffer A-soluble glutelin. Panel B: The immunoblot profiles of panel A. The arrowhead at the right shows the MG60 subunit.

tion of corresponding proteins, although the action of end-proteases is not negligible. For the purpose of establishing intimate linkages between the MG60-like glutelin and GBSS proteins, the isolation of cDNA encoding all of this family of proteins will provide some information to resolve the problem of similarity or dissimilarity.

REFERENCES

- 1) Takumi K, Udaka J, Kanoh M, Koga T, Tsuji H. 1996. Polypeptide compositions and antigenic homologies among prolamins from Italian, Common and Japanese millet cultivars. *J Sci Food Agric* **72**: 141-147.
- 2) Milfin BJ, Shewry PR. 1979. The biology and biochemistry of cereal seed proteins. In: *Seed Protein Improvement in Cereals and Legumes*, Vol. 1, p 137. IAEA, Vienna.
- 3) Matsudaira P. 1987. Sequence from picomole quantities of protein electroblotted onto polyvinylidene difluoride membranes. *J Biol Chem* **262**: 10035-10038.
- 4) Krishnann HB, Okita TM. 1986. Structural relationships among rice glutelin polypeptides. *Plant Physiol* **81**: 748-753.
- 5) O'Farrell PZ, Goodman HM, O'Farrell PH. 1977. High resolution, two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**: 1133-1142.
- 6) Nelson OE, Rins HW. 1962. The enzymatic deficiency in the *waxy* mutant of Maize. *Biochem Biophys Res Comm* **9**: 297-300.
- 7) Echt GS, Schwartz D. 1981. Evidence for the inclusion controlling elements within the structural gene at the *waxy* locus in maize. *Genetics* **99**: 275-284.
- 8) Shannon JC, Garwood DL. 1984. Genetics and physiology of starch development. In: *Starch: Chemistry and Technology*, 2nd ed, (Whistler RL, BeMiller JN, Paschal EF, eds), p 25-86. Academic Press, New York.
- 9) Vos-Scheperkeuter GH, Boer WD, Visser RGF, Feenstra WJ. 1986. Identification of granule-bound starch synthase in potato tubers. *Plant Physiol* **82**: 411-416.

Fig. 3. Immunoblotting using anti-MG60 for cross-reactive buffer A-soluble MG60-like glutelin components in rice and other starch-storing cereal grains. Panel A: Lanes: 1, rice; 2, barley; 3, corn; 4, wheat; 5, rye; 6, oat. The numbers on the right represent MMs of marker proteins in kDa. Panel B: Immunoblot profiles of panel A. The arrowhead at the left shows MG60-like glutelin components.

- 10) Clark JR, Robertoson M, Ainsworth CC. 1991. Nucleotide sequence of a wheat (*Triticum aestivum* L.) cDNA clone encoding the waxy protein. *Plant Molecular Biol* **16**: 1099–1101.
- 11) Sano Y. 1984. Differential regulation of waxy gene expression in rice endosperm. *Theor Appl Genet* **68**: 467–473.
- 12) Hirano H, Sano Y. 1991. Molecular characterization of the waxy locus of rice (*Oryza sativa*). *Plant Cell Physiol* **32**: 989–997.
- 13) Shure M, Nessler S, Fedrof N. 1983. Molecular identification and isolation of the waxy locus in maize. *Cell* **35**: 225–233.
- 14) Van der Leij FR, Visser RGE, Ponsein AS, Jacobsen E, Feenstra WJ. 1991. Sequence of the structural gene for granul-bound starch synthase of potato (*Solanum tuberosum* L.) and evidence for a single point deletion in the amf allele. *Mol Gen Genet* **228**: 240–248.
- 15) Towin H, Staelin T, Goardon J. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4351–4354.
- 16) Takumi K, Koga T, Kanoh M, Udaka J, Tsuji H, Manabe H. 1995. Immunochemical cross-reactivity between globulins from buckwheat and indigo seeds. *Biosci Biotech Biochem* **59**: 1971–1972.
- 17) Preiss J. 1988. Carbohydrates: Structure and function. In: the Biochemistry of Plants, Vol 14. (Preiss L, ed), p 181–254. Academic Press, New York.
- 18) Peterson DM. 1978. Submit structure and composition of oat seed globulin. *Plant Physiol* **62**: 506–509.