JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Amino Acid Variation in the 10 kDa *Oryza* Prolamin Seed Storage Protein

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The deduced amino acid variability for the 10 kDa prolamin was determined for 16 *Oryza* species, both cultivated (rice) and wild. Prolamin, a seed storage protein and site of nitrogen and sulfur accumulation, is sequestered in the subaleurone layer of the starchy endosperm for use during seedling germination. The 10 kDa prolamin amino acid distribution for the cultivated species (*O. sativa* and *O. glaberrima*) was determined and compared to those of wild and, hitherto unknown, noncultivated *Oryza* species. Four wild species (*O. granulata*, *O. australiensis*, *O. brachyantha*, and *O. meyeriana*) exhibited the greatest residue heterogeneity in both the signal and mature peptide regions. A breakdown of the essential amino acid variance among three Central/South American and one African endemic wild species is also presented and compared with those of rice.

KEYWORDS: ***Oryza*; rice; prolamin; amino acid variation; protein; DNA**

INTRODUCTION

Cereals comprise the most important food crops in the world in terms of production and consumption and provide a major source (>60%) of the overall calories and protein for many Asian countries (*1*). Rice (*Oryza sativa*) is one of the most commonly consumed cereal grains, providing >75% of our food at the international level. The global, annual rice production is 562,260 thousand metric tons (tmt), a yield that is a close third to wheat (584,874 tmt) and maize (576,821 tmt) (*2*). However, unlike wheat and corn, rice is consumed almost exclusively by humans. Consequently, considerable economic and agricultural importance has underscored the need to improve the protein quality of this crop, as compared to that of wheat and corn.

A major concern among nutritionists is that human protein deficiencies arise because a sufficient "bulk" of grain cannot be consumed to meet dietary protein needs, especially in young children (*1*, *3*) and women (*4*). The protein in rice is packaged within protein bodies (PB-1 and PB-2) that reside in the subaleurone layer of the starchy endosperm (*3*). This study focuses on a group of these proteins, specifically the 10 kDa prolamin fraction of the rice seed storage protein.

Prolamin is a seed storage protein that is unique to the grass family; its primary function is the accumulation of nitrogen and sulfur, two elements required for seed germination (*5*). Prolamin is distinguished by its alcohol solubility from the three other classes of seed storage proteins: the albumins (water-soluble), globulins (salt-soluble), and glutelins (dilute alkali- or acid-soluble) (*6*). Of these four categories of proteins, glutelins and prolamins are the most abundant in cereal crops, comprising nearly 90% of its total seed protein (*7*). Prolamin is the main type of storage protein found in cereal crops, with the exception of rice and oats. Between 30 and 60% of the total protein content in maize, sorghum, barley, and wheat consists of prolamin (*1*). The primary storage protein in rice is a glutelin-like protein; prolamin is found in lower quantities. The specific amount of rice prolamin that is extracted, however, is dependent upon the extraction techniques used, and as a result, values reported over the past decade have fluctuated: 5% (*8*), 20−25% (*9*), 18% (*10*), and 35% (*11*).

Prolamin protein is sequestered within a type 1 protein body (PB-1) in the starchy endosperm of the rice grain (*12*, *13*). PB-1 cannot be easily digested in the human body because it is resistant to the action of proteolytic enzymes such as pepsin (*13*). The signal peptide region by which it is believed that the 10 kDa prolamin is routed to PB-1 has been identified in cultivated rice (*14*). The need for a more detailed understanding of the molecular diversity and mechanism of protein body deposition has been identified as an existing barrier to crop improvement (*15*).

Recent efforts have elucidated the diversity of, and relatedness among, wild and domesticated species at the DNA level for the *Oryza* genus (*16*, *17*) using several genetic markers. However, amino acid variation in the prolamin of wild *Oryza* species (20 of the 22 species) is not yet known. Our principal objective was to determine the amino acid composition and identify the extent and pattern of heterogeneity of the 10 kDa prolamin residue in *Oryza* for use in genetic manipulation and molecular breeding. We placed special emphasis on the wild species because they are rich sources of genetic variability.

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10.1021/jf0305480 CCC: $27.50
Published on Web 03/25/2004

Table 1. *Oryza* Species Examined for Residue Composition

cultivated *Oryza* species (genome designation)	uncultivated *Oryza* species (genome designation)[a]
O. sativa (*AA*)	*O. alta* (*CCDD*), *IRRI 101395*
O. glaberrima (*AA*), *PI 450198*	*O. australiensis* (*EE*), *IRRI 101144*
	O. brachyantha (*FF*), *IRRI 105172*
	O. eichingeri (*CC*), §
	O. grandiglumis (*CCDD*), *IRRI 101405*
	O. granulata (*GG*), *IRRI 106468*
	O. latifolia (*CCDD*), *IRRI 100165*
	O. meridionalis (*AA*), *IRRI 101145*
	O. meyeriana (*GG*)
	O. minuta (*BBCC*), *IRRI 101082*
	O. nivara (*AA*), *USDA PI 590404*
	O. punctata (*BB*), §
	O. rhizomatis (*CC*), *IRRI 103410*
	O. rufipogon (*AA*), *PI 590418*

[a] IRRI, International Rice Research Institute; PI, Plant Introduction Number of the U.S. Department of Agriculture; §, DNA obtained from Dr. Y. Sano, Hokkaido University, Japan.

MATERIALS AND METHODS

Plant Growth. Seed samples of 16 *Oryza* species (**Table 1**), supplied by the International Rice Research Institute (IRRI) in the Philippines, or by the U.S. Department of Agriculture (USDA), were germinated on moist filter paper and then transplanted into submerged pots in a greenhouse enclosure. Details of the *Oryza* plant growth conditions have been described elsewhere (*17*).

Genomic DNA Extraction and Amplification. DNA was isolated from leaf material using a modification (*18*) of the CTAB extraction method of Saghai-Maroof (*19*). Two sets of PCR primers were used to amplify the gene that encodes the 10 kDa prolamin protein, as described (*17*). In most cases, the primers PR10.1F2 (5′ ACG TGA ATT CCA CCA TCT GGA ATC TTG 3′) and PR10.3RV (5′ ACG TTC TAG AAG TGT TTG CAC ACG ATA GTA 3′) were used in amplification. When these primers did not amplify, PR10.1E (5′ ACG TGA ATT CAT GGC AGC ATA CAC CAG CAA G 3′) and PR10.2RB (5′ ACG TGG ATC CAA CCA CAG GAA GAG AGT TGG 3′) were used (*20*). The latter primers are located 2 base pairs downstream of the 5′ and 3′ ends, resulting in a truncated sequence product.

PCR Product Sequencing. Approximately 30−60 ng of column-cleaned PCR product (Quiagen, Valencia, CA) was used to prepare half-volume sequencing reactions using the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase FS in the ABI Big Bye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Foster City, CA) with an annealing temperature of 50 °C. Samples were prepared according to the manufacturer's protocol and alcohol precipitated with either an ethanol/sodium acetate or 95% 2-propanol protocol, following Perkin-Elmer protocols. DNA was sequenced at the University of Maine (Orono, ME), University of Florida (Gainesville, FL), and Virginia Tech (Blacksburg, VA) sequencing facilities.

Sequence Data Analysis. Sequences were manually edited and aligned using the Sequence Navigator program (Applied BioSystems, Perkin-Elmer), given the unambiguous nature of the data. Both forward and reverse sequences were examined for the presence of multiple gene species.

Deduced amino acid residues were obtained for each of the nucleotide sequences using the Sequence Navigator software package. A GenBank acquisition for the deduced amino acid sequence of the 10 kDa *O. sativa* prolamin polypeptide (*21*) was used as a reference (accession no. X17074) to confirm and validate the *O. sativa* accession used in this study.

RESULTS

Signal Peptide Region in the 10 kDa Prolamin. A 24 amino acid signal peptide precedes the mature prolamin peptide, with a consensus sequence of MAAYTSKIFALFALIALSASATTA (*21*). This region is thought to be removed prior to protein packaging within PB-1 (*22*). Among the 16 species examined, 4 species (*O. granulata*, *O. australiensis*, *O. brachyantha*, and *O. meyeriana*) exhibited the greatest amino acid residue variability within the signal peptide region, relative to *O. sativa* (**Figure 1** and **Table 2**). Ambiguous nucleotide residues led to incomplete amino acid deduction in *O. grandiglumis*, *O. brachyantha*, *O. punctata*, and *O. granulata*. The region of greatest variability occurred between residues 10 and 29.

Mature Peptide Region in the 10 kDa Prolamin. The mature peptide consists of 110−111 amino acid residues. The consensus sequence for the mature peptide in cultivated *O. sativa* is ITTMQYFPPTLAMGTMDPCRQYMMQTLGMG-SSTAMFMSQPMALLQQQCCMQLQGMMPQCHCGTSC-QMMQSMQQVICAGLGQQQMMKMAMQMPYMCNMAP-VNFQLSSCGCC (*21*). *O. sativa* and *O. glaberrima* are identical in their residue constitutions (**Figure 1** and **Table 3**). Premature 3′ end sequence termination due to primer location occurred in *O. australiensis*, *O. meyeriana*, *O. latifolia*, *O. rhyzomatis*, and *O. granulata*.

Due to the lack of complete sequence coverage in all species, an emphasis was placed on species that contained a greater than average (relative to the cultivated species *O. sativa*) representation of amino acids. Those species are (1) *O. granulata* (> alanine, arginine, glutamate, asparagine, and valine), (2) *O. australiensis* (> alanine, arginine, glutamate, isoleucine, and valine), (3) *O. brachyantha* (> alanine, asparagine, proline, and tyrosine), and (4) *O. meyeriana* (> alanine, arginine, glutamate, asparagine, and valine). A comparison of these wild species to *O. sativa* indicated that the greatest variability occurs between residues 85 and 110.

Additionally, three Central/South American and one African endemic *Oryza* species exhibited greater than average content of several essential amino acids. The Central/South America species are *O. alta* (> leucine, methionine, and threonine), *O. grandiglumis* (Brazilian accession: > leucine, methionine, and threonine), and *O. latifolia* (Guatemalan accession: > lysine, methionine, and threonine). *O. punctata* (> leucine, methionine, and threonine) is found only in two isolated regions of central and southeastern Africa. These four species, however, exhibit very little overall residue heterogeneity in either the signal or mature peptide regions when compared with cultivated rice (**Figure 1**).

DISCUSSION

Although our understanding of the exact pathway by which prolamin is deposited into its storage body has not been fully elucidated, the work of several research groups has provided targets through which it may be possible to reallocate protein stored in PB-1 to PB-2. Masamura et al. suggested that the targeting of prolamin to PB-1 occurs with the interaction of the signal peptide with the PB membrane (*14*). A later comparison of the signal peptides for proteins sequestered in PB-1 and PB-2 highlighted the unique nature of each of the sequences, supporting the notion that they may be responsible for specific sorting into the protein bodies (*23*). A molecular chaperone hypothesis has also been explored by Li et al. (*24*, *25*) and Meunch et al. (*26*) using BiP, a protein located in the ER lumen that retains prolamin by promoting its folding and assembly into PB-1.

With this in mind, the data presented here provide the potential for crop improvement through standard molecular breeding and manipulation of the prolamin fraction itself. For example, species with greater natural stores of certain essential

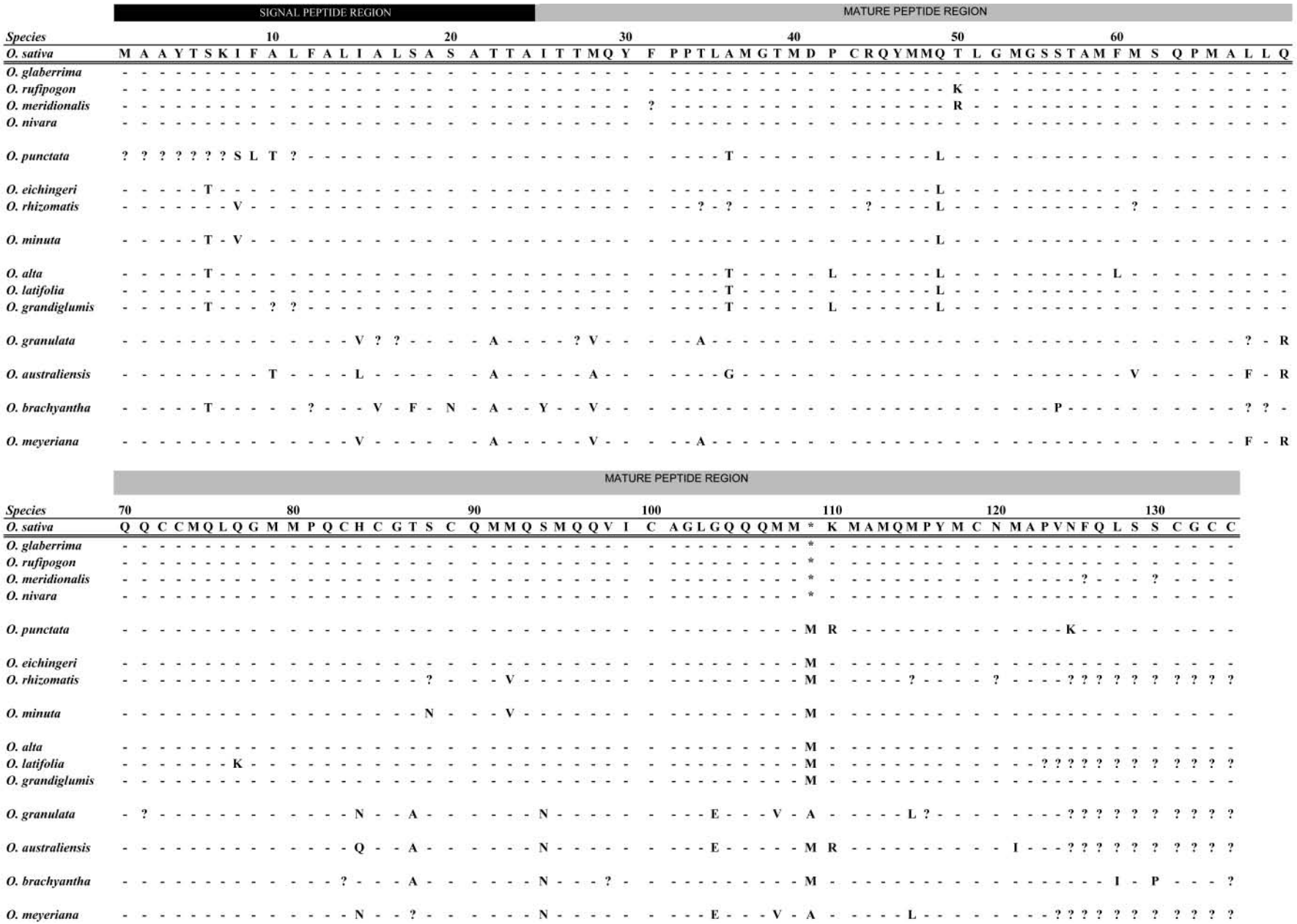

Figure 1. Deduced residue content for 16 wild and cultivated *Oryza* species. Ambiguous amino acid residues are indicated by a "?", like residues relative to *O. sativa* are indicated by a "–", and gaps are indicated by a "∗". The signal and mature peptide regions are highlighted above the residues.

Table 2. Deduced Amino Acid Residue Variation in the Signal Peptide Region of the 10 kDa Prolamin Protein in 16 *Oryza* Species[a]

	amino acid																		
species	A	R	Q	E	G	H	L	K	M	F	T	W	N	D	C	I	P	S	Y	V
Oryza alta	8						3	1	1	2	4					2		2	1	
O. australiensis	8						4	1	1	2	3					1		3	1	
O. eichingeri	8						3	1	1	2	4					2		2	1	
O. glaberrima	8						3	1	1	2	3					2		3	1	
O. grandiglumis[b]	7						2	1	1	2	4					2		2	1	
O. meridionalis	8						3	1	1	2	3					2		3	1	
O. meyeriana	9						3	1	1	2	2					1		3	1	1
O. brachyantha[b]	8						3	1	1	2	3		1			2			1	1
O. latifolia	8						3	1	1	2	3					2		3	1	
O. minuta	8						3	1	1	2	4					1		2	1	1
O. nivara	8						3	1	1	2	3					2		3	1	
O. punctata[b]	5						3			1	3					1		3		
O. rhizomatis	8						3	1	1	2	3					1		3	1	1
O. rufipogon	8						3	1	1	2	3					2		3	1	
O. sativa	8						3	1	1	2	3					2		3	1	
O. granulata[b]	8						2	1	1	2	2					1		3	1	1
mean	7.8						2.9	1	1	1.9	3.1		1			1.6		2.7	1	1
SD	0.8						0.4	0	0	0.3	0.6		0			0.5		0.5	0	0

[a] Residue abbreviations follow standard IUPAC nomenclature: (A) alanine, (R) arginine, (N) asparagine, (D) aspartate, (S) cysteine, (E) glutamate, (Q) glutamine, (G) glycine, (H) histidine, (I) isoleucine, (L) leucine, (K) lysine, (M) methionine, (F) phenylalanine, (P) proline, (S) serine, (T) threonine, (Y) tyrosine, (W) tryptophan, and (V) valine. [b] Nucleotide sequence has unscored/ambiguous bases.

Table 3. Deduced Amino Acid Distribution for the Mature Prolamin Peptide in the 16 *Oryza* Species Examined in This Study[a]

	amino acid																		
species	A	R	Q	E	G	H[b]	L[b]	K[b]	M[b]	F[b]	T[b]	N	D	C[b]	I[b]	P	S	Y	V[b]
O. sativa	***6***	***1***	***19***	***0***	***8***	***1***	***7***	***1***	***22***	***3***	***7***	***2***	***1***	***11***	***2***	***7***	***7***	***3***	***2***
O. alta	5	1	18	0	8	1	10	1	23	2	8	2	1	11	2	6	7	3	2
O. australiensis[c]	7	3	18	1	7	0	5	0	20	3	6	2	1	8	3	7	4	3	3
O. eichingeri	6	1	18	0	8	1	8	1	23	3	7	2	1	11	2	7	7	3	2
O. glaberrima	6	1	19	0	8	1	7	1	22	3	7	2	1	11	2	7	7	3	2
O. grandiglumis	5	1	18	0	8	1	9	1	23	3	8	2	1	11	2	6	7	3	2
O. meridionalis	6	2	19	0	8	1	7	1	22	1	6	2	1	11	2	7	6	3	2
O. meyeriana[c]	8	2	17	1	6	0	6	1	19	3	5	3	1	8	2	7	4	3	3
O. brachyantha	7	1	19	0	8	1	4	1	22	3	6	3	1	9	2	9	4	4	2
O. latifolia[c]	5	1	16	0	7	1	7	2	23	2	8	1	1	8	2	6	5	3	1
O. minuta	6	1	18	0	8	1	8	1	22	3	7	3	1	11	2	7	6	3	3
O. nivara	6	1	19	0	8	1	7	1	22	3	7	2	1	11	2	7	7	3	2
O. punctata	5	2	18	0	8	1	8	1	23	3	8	1	1	11	2	7	7	3	2
O. rhizomatis[c]	5	0	17	0	7	1	7	1	20	2	6	0	1	8	2	7	4	3	3
O. rufipogon	6	1	19	0	8	1	7	2	22	3	6	2	1	11	2	7	7	3	2
O. granulata[c]	9	2	16	1	6	0	6	1	19	2	4	3	1	8	2	6	4	3	4
mean	6.13	1.31	18	0.19	7.56	0.82	7.06	1.06	21.6	2.60	6.63	2.0	1.0	9.93	2.06	6.90	5.81	3.06	2.3
SD	1.15	0.7	1.03	0.40	0.73	0.40	1.44	0.44	1.40	0.62	1.15	0.82	0.0	1.43	0.25	0.71	1.57	0.25	0.70

[a] Standard IUPAC abbreviations used, as described in **Table 1**. [b] Essential amino acids: H, L, K, M, F, T, C, I, W, and V must be supplied through diet. [c] Premature sequence termination due to primer location.

(not supplied through diet) amino acids, relative to *O. sativa*, such as *O. alta*, *O. latifolia*, *O. grandiglumis*, and *O. punctata* (**Table 3**), may be cultivated and/or bred with current crop varieties to supply desirable genetic backgrounds. Additionally, repackaging scenarios that sequester the 10 kDa prolamin into a more digestible protein body (PB-2) are broadened to include quantity upgrades. The efficient utilization of reallocated protein in the plant may not only improve the overall protein content of rice but may also minimize issues of decreased yield because no additional nitrogen is required.

Alternatively, species that exhibit mutations in the signal or mature portions of the peptide may exhibit no, or poorly formed, prolamin, thereby naturally optimizing the production of the glutelin fraction. Indeed, although the extractability of prolamin in cultivated rice has been examined, the extractability of prolamin in wild *Oryza* species is unknown. Further work examining the endogenous prolamin levels in species with mutated signal peptide regions (*O. australiensis*, *O. brachyantha*, *O. granulata*, and *O. meyeriana*) may indicate that these species have naturally occurring low levels of prolamin synthesis. Our laboratory is currently exploring this hypothesis.

Beyond the amino acid composition, the digestibility of proteins in foods after cooking and ingestion is of prime importance to nutritionists. Rice is the only cereal in which protein digestibility decreases with cooking (*3*). In humans, it has been demonstrated that PB-1 remains intact in human feces following the cooking process and digestion (*13*, *27*). Factors affecting digestibility include amino acid sequence in the peptide side chain, spatial configurations, and cross-linkages (*1*). It has been further argued that improvement of digestibility is the best way to increase available protein without additional inputs but that this can be done only with the knowledge of the amino acid sequence (*1*). One of the most widely used methods to evaluate plant protein is the Protein Digestibility-Corrected

Amino Acid Score (PDCAAS) (*28*), which considers three parameters to evaluate protein quality: essential amino acid profile, digestibility, and its ability to supply essential amino acid to humans (*28*−*30*). Here, we describe one of the components of digestibility, the amino acid sequence, which may be used to improve the digestibility of rice protein.

SAFETY

The experiments presented here comply with current U.S. laws regarding the safe usage and handling of chemicals and reagents.

ABBREVIATIONS USED

kDa, kilodalton; DNA, deoxyribosenucleic acid; tmt, thousand metric ton; USDA, U.S. Department of Agriculture; IRRI, International Rice Research Institute; PCR, Polymerase Chain Reaction; bp, base pair; SD, standard deviation; ng, nanogram; PB-1, protein body 1; PB-2, protein body 2.

ACKNOWLEDGMENT

Seed materials were kindly provided by the International Rice Research Institute (IRRI) and the U.S. Department of Agriculture, Agriculture Research Service, National Plant Germplasm System. We thank Dr. Yoshio Sano, Hokkaido University, Japan, and Dr. Christoph Neihous, University of Bonn, for supplying plant material and DNA samples.

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Received for review July 30, 2003. Revised manuscript received January 14, 2004. Accepted January 29, 2004. This work was supported by grants from the Virginia Academy of Science, *Sigma Xi*, the Graduate Research Development Project, The Department of Biology at Virginia Tech, the Jeffress Foundation, and the CARUDO Foundation.

JF0305480