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ARTICLE

Molecular Cloning, Mass Spectrometric Identification, and Nutritional Evaluation of 10 Coixins in Adlay (*Coix lachryma-jobi* L.)

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Adlay (*Coix lachryma-jobi* L. var. ma-yuen Stapf) is regarded as a nutritive food source as well as herbal medicine. The food nutrition is a consequence of its high protein content and superior amino acid composition. From ca. 200 expressed sequence tag (EST) sequences in maturing adlay grains, clones encoding precursor polypeptides of 10 seed storage proteins in the prolamin family, including 8 α-coixin isoforms, 1 δ-coixin, and 1 γ-coixin, were identified. Full-length cDNA fragments encoding these 10 coixins were obtained by PCR cloning. Mass spectrometric analyses confirmed the presence of these 10 coixins in the extract of adlay grain. Calculated amino acid compositions indicate that all 10 coixins are rich in glutamine (>20% in α-coixin isoforms, 13.3% in δ-coixin, and 31.2% in γ-coixin). The 8 α-coixin isoforms are low in methionine, cysteine, and lysine (on average, 0.8, 0.6, and 0.1%, respectively). However, the δ-coixin is a sulfur-rich protein (18.2% methionine and 9.1% cysteine), and the γ-coixin is a nutritive protein composed of 2.0% methionine, 6.6% cysteine, 2.6% lysine, and 8.9% histidine. The company of δ-coixin and γ-coixin with α-coixin isoforms enhances the nutritional value of alday grain for human consumption.

KEYWORDS: Adlay; coixin; glutamine; storage protein; sulfur-rich

INTRODUCTION

Since the documentation of *Pen-Tsao-Kang-Mu*, an ancient Chinese medical book published in 1596, adlay (soft-shelled Job's tears, *Coix lachryma-jobi* L. var. ma-yuen Stapf) grain has long been consumed in Asian countries as a nourishing food source as well as herbal medicine for its nutritional value and functional effects (*1*−*3*). Numerous publications, mostly emphasizing medicinal effects, have shown that several constituents found in adlay grain are beneficial to human health. For example, lactams isolated from adlay bran were found to inhibit lung and colon cancer cells in vitro (*2*); hydroxy unsaturated fatty acids isolated from adlay grain, such as 9-hydroxy-(10*E*,12*E*)-octadecadienoic acid, were demonstrated to act as natural ligands against peroxisome proliferator-activated receptor γ (*4*); and phenolic components extracted from adlay grain possessed antioxidative and anti-inflammatory activities against Cu^{2+}-treated low-density lipoprotein oxidation and lipopolysaccharide-induced inflammation in RAW 264.7 macrophages (*5*). In contrast, much less investigation has been done on the nutritional value of adlay grain.

Similar to other cereal grains, adlay grain mainly comprises starch (approximately 60%). The nutritional value of adlay grain is attributed to its significantly rich oil content and slightly high protein content among cereal grains; the oil and protein contents in adlay grains are approximately 10 and 15%, whereas those in rice, barley, and wheat are 1.9−2.3 and 8−12%, respectively (*6*). In addition, the nutritional value of adlay grain is also a consequence of its superior amino acid composition with respect to human requirement as amino acid compositions of proteins from most plant food sources are generally short in methionine, cysteine, and lysine (*7*). The major proteins found in adlay grain are prolamins, which represent approximately 60% of its total endosperm proteins (*8*). Prolamins, usually soluble only in alcohol solutions, are a group of storage proteins found in cereal grains and other members of the grass family (*9*). Generally, unique names are given for prolamins in different cereal grains other than rice, for example, gliadin, hordein, secalin, zein, avenin, and coixin for homologous prolamins found in wheat, barley, rye, maize, oat, and adlay, respectively. Because of their importance in nutrition and food processing, cereal prolamins have been actively investigated in recent years (*10*, *11*).

A large number of protein sequences deduced from either genomic DNA sequences or expressed sequence tag (EST) clones have provided rich sources to identify proteins by peptide mass fingerprinting (*12*). Moreover, the utilization of liquid chromatography−electrospray ionization−tandem mass spectrometry

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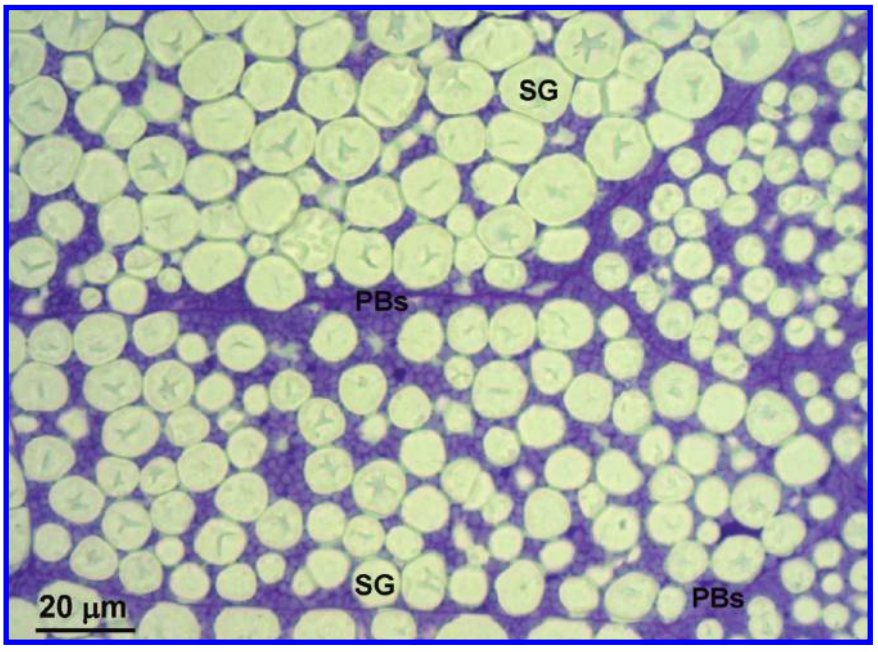

Figure 1. Light microscopy of endosperm cells in adlay grain. The abundant large particles (light color) of 5−20 μm are starch granules (SG), and the numerous small particles (purple) of 1−3 μm are protein bodies (PBs).

Table 1. Summary of the Full-Length cDNA Fragments Encoding Coixins

coixin	accession no.	cDNA length (bp)	open reading frame (bp)	amino acid residues	protein mass (kDa)	p*I*
α-1	FJ669134	948	795 (52−846)	264	26.5	8.02
α-2	FJ669135	978	798 (54−851)	265	26.6	7.41
α-3	FJ669136	942	798 (31−828)	265	26.4	8.93
α-4	FJ669137	941	801 (47−847)	266	26.7	8.85
α-5	FJ669138	1097	942 (49−990)	313	31.6	8.94
α-6	FJ669139	852	594 (59−652)	197	19.3	7.41
α-7	FJ669140	1107	876 (45−920)	291	30.3	8.8
α-8	FJ669141	913	729 (57−785)	242	24.4	8.33
γ	FJ669133	1173	969 (70−1038)	322	35.9	6.98
δ	FJ669142	688	501 (51−551)	166	15.6	7.69

(LC-ESI-MS/MS) has powerfully assisted protein identification and characterization (*13*). For a comprehensive understanding of coixins at the molecular level, we sequenced approximately 200 EST clones from maturing adlay grain. Putative fragments encoding 10 coixins were identified in these EST clones. Corresponding proteins of the identified clones were subsequently identified in the extract of adlay grains by LC-ESI-MS/MS analyses. The nutritional value of these coixins in terms of their amino acid compositions was evaluated.

MATERIALS AND METHODS

Plant Materials. Mature and fresh maturing adlay (*C. lachryma-jobi* L. var. ma-yuen Stapf) grains were grown and harvested at the Crop Improvement Department, Taichung District Agricultural Improvement Station. Mature grain was used for the preparation of crude protein extract, and maturing grain approximately 20−30 days after pollination was used to extract mRNA for construction of a cDNA library.

Light Microscopy of Adlay Grain. Adlay grain was immersed in water overnight and cut into 1.5−2.0 mm sections in fixative solution. Specimens were fixed in a solution of 4% (v/v) glutaraldehyde in 0.1 N sodium phosphate buffer, pH 6.8, dehydrated in an alcohol series, and then embedded in LR white resin (Sigma). Tissues were infiltrated with 100% London white resin at 4 °C for 30 days before polymerization at 60 °C for 24 h in an oxygen-free environment. Sections (800 nm) were stained with 0.1% toluidine blue O for general histological examination. Digital images were captured by a CCD camera (AxioCam, ICc3, Carl Zeiss AG) through a Carl Zeiss Axioplan microscope.

Isolation of Poly(A)$^+$ RNA and cDNA Library Construction. Total RNA was extracted from maturing adlay grain ground in liquid nitrogen using the phenol/SDS method (*14*). Poly(A)$^+$ RNA, isolated with Dynabeads (Dynal) following the manufacturer's instructions, was dissolved in diethyl pyrocarbonate-treated water and quantified as the absorbance at 260 nm with a spectrophotometer. cDNA was synthesized from poly(A)$^+$ RNA according to the protocol described in the manufacturer's instructions (cDNA synthesis, ZAP-cDNA synthesis, and ZAP-cDNA Gigapack III Gold Cloning kits purchased from Stratagene). A cDNA library of approximately 10^6 plaques was constructed with 5 μg of poly(A)$^+$ RNA. Mass excision was performed to convert the phage library into bacterial pBluescript libraries.

In-House Generated EST Sequencing and Sequence Analyses. Bacterial libraries were sprayed and grown on LB plates with antibiotic ampicillin. Colonies were selected at random, and recombinant cDNA plasmids were prepared using the Minipre Purification kit (Protech) prior to EST sequencing by the dideoxy chain termination method with the BigDye terminator cycle sequencing kit and ABI 377 DNA sequencer (Perkin-Elmer). To maximize the possibility of detecting coding regions in the databases, 5′ ends of cDNA inserts were first sequenced using T3 primer, 5′-AATTAACCCTCACTAAAGGG-3′. Approximately 200 EST clones derived from the cDNA libraries were sequenced and analyzed by the Blast program (http://www.ncbi.nlm.nih.gov/blast/) at the National Center for Biotechnology Information (*15*).

The EST clones showing homology to seed storage proteins were collected. The cDNA fragments larger than 0.5 kb were completely sequenced from both ends using T3 and T7 primers. N-terminal signal sequence responsible for ER targeting was predicted using the SignalP

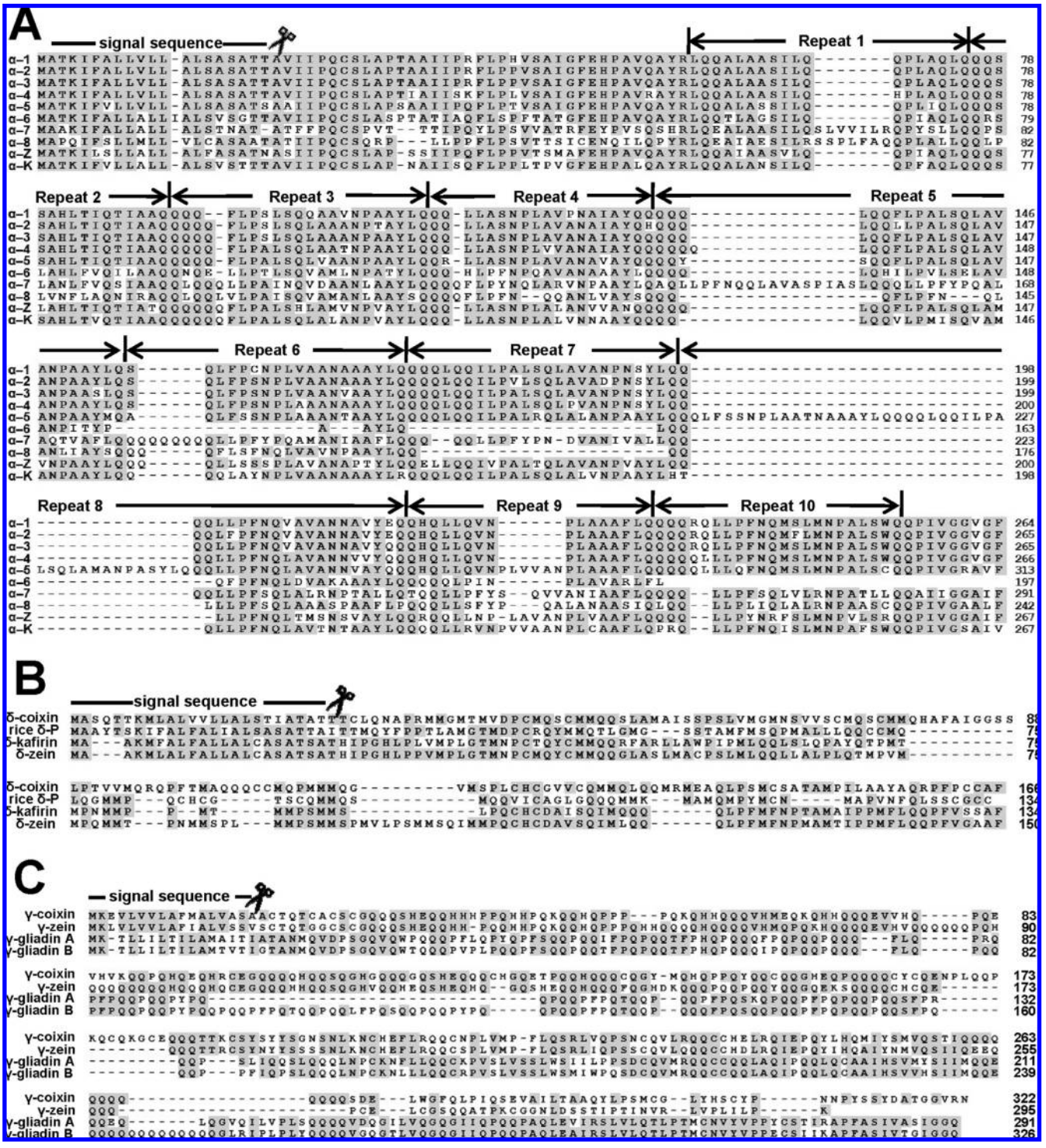

Figure 2. Sequence alignment of the precursor polypeptides of (**A**) 8 α-coixin isoforms, α-zein (accession no. P04698) and α-kafirin (accession no. P14691); (**B**) δ-coixin, rice δ-prolamin (accession no. CAA59142), sorghum δ-kafirin (accession no. AAK72689), and maize δ-zein (accession no. AAA33541); and (**C**) γ-coixin, maize γ-zein (accession no. AAL16979), and wheat γ-gliadins (accession no. ACJ03439 and ACJ03534 for γ-gliadins A and B, respectively). The amino acid number for the last residue in each line is listed on the right for each protein. Broken lines in the sequences represent gaps introduced for best alignment. The cleavage site of the putative N-terminal signal sequence is indicated by a scissors symbol.

program (http://www.cbs.dtu.dk/services/SignalP/) in the World Wide Web Prediction Server Center for Biological Sequence Analysis (*16*). Sequence alignments were executed by the CLUSTAL W program at Pôle Bioinformatique Lyonnais (PBIL) (Lyons, France).

Protein Analysis of the Crude Extract of Adlay Grain by SDS-PAGE. Proteins were extracted by homogenizing adlay grain with 1 mL of 12.5 mM sodium borate, pH 10, 1% SDS, and 2% β-mercaptoethanol using a Polytron. For SDS-PAGE analysis, the crude extract was mixed with the sample buffer containing 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 0.02% bromophenol blue, and 10% glycerol with β-mercaptoethanol according to the Bio-Rad instruction manual. To resolve the proteins extracted from mature adlay grain, the separating gel was composed of 15% polyacrylamide, and the electrophoresis was performed under 120 V for 100 min. Following electrophoresis, the gel was stained with Coomassie Blue R-250.

LC-ESI-MS/MS Identification. All of the visible protein bands resolved in the SDS-PAGE gel were manually excised and ground into

pieces. The pieces were reduced with 10 mM DTT for 45 min at 56 °C and alkylated with 55 mM iodoacetamide for 30 min in the dark at 25 °C. Following dehydration in acetonitrile, the pieces of protein bands were dried and digested with 0.1 μg of chymotrypsin (Roche) overnight at 25 °C. The supernatant containing chymotryptic peptides was combined with two more extracts of the gel by 50% acetonitrile/1% trifluoroacetic acid and subjected to LC-ESI-MS/MS analysis on a Q-TOF Ultima API (Micromass, Manchester, U.K.) instrument for protein identification. Raw data files were processed by ProteinLynx 2.2 and converted into pkl files for searching against the Swiss-Prot protein sequence database using Mascot software (Matrix Science Ltd., London, U.K.).

RESULTS

Light Microscopy of Endosperm Cells of Adlay Grain. Light microscopy showed that endosperm cells in adlay grain were predominantly packed with starch granules of 5−20 μm (**Figure 1**). Besides, numerous protein bodies of 1−3 μm were also found densely accumulated around starch granules. The abundance of starch granules and protein bodies observed in the microscopy was approximately in agreement with the composition (60% starch and 15% protein) of adlay grain reported previously (*7*).

Identification of Genes Encoding Coixins from EST Sequences of Maturing Adlay Grain. The presence of seed storage proteins, prolamin (coixin) isoforms, in adlay grain was verified by identification of their corresponding EST clones prepared with maturing grain. Ten groups putatively encoding 8 α-coixin isoforms, 1 δ-coixin, and 1 γ-coixin were identified in the approximately 200 EST clones. Properties of full-length cDNA sequences encoding precursor polypeptides of these 10 coixins, including GenBank accession numbers, nucleotide residues of cDNA fragments, open reading frames, amino acid residues of the deduced precursor polypeptides, molecular masses of the deduced mature proteins, and isoelectric points, are summarized in **Table 1**.

Sequence Analysis of the 10 Deduced Coixins. All precursor polypeptides of the 10 deduced coixins contained a cleavable N-terminal signal sequence for ER targeting via a signal recognition particle dependent pathway. Sequence alignment among the eight α-coixins showed that sequence identity was >90% among α-coixin 1, 2, 3, and 4 isoforms (**Figure 2A**). In comparison with the known α-coixin sequences, three deduced α-coixins (accession no. X57831, X57832, and X61113) are equivalent to α-coixin 1, 2, and 3 isoforms of this study, respectively (*17*, *18*). Highly homologous proteins of δ-coixin, for example, δ-prolamin, δ-kafirin, and δ-zein, were found in rice, sorghum, and maize, respectively (**Figure 2B**). Homologous proteins of γ-coixin with relatively low sequence identity, for example, γ-zein and γ-gliadins, were found in maize and wheat, respectively (**Figure 2C**). Relatively low sequence homology (13−19 or 15−17%) was observed between α-coixins and δ-coixin or γ-coixin.

Detection of the 10 Coixins in Mature Adlay Grain by LC-ESI-MS/MS. To confirm the presence of the 10 coixins in adlay, total seed proteins were extracted from mature grain and resolved by SDS-PAGE (**Figure 3**). All of the visible protein bands resolved in the SDS-PAGE gel were subjected to LC-ESI-MS/MS analysis (**Table 2**). The results indicated that all 10 coixins were detected in the extract of adlay grains, and their resultant abundance represented approximately 60% of total grain proteins.

Ananlyses of Calculated Amino Acid Compositions of the 10 Coixins. Amino acid compositions calculated from the deduced sequences (**Table 3**) indicate that all 10 coixins are rich in glutamine (>20% in α-coixin isoforms, 13.3% in δ-coixin, and 31.2% in γ-coixin). The 8 α-coixin isoforms belong to common seed storage proteins, that is, short in methionine, cysteine, and

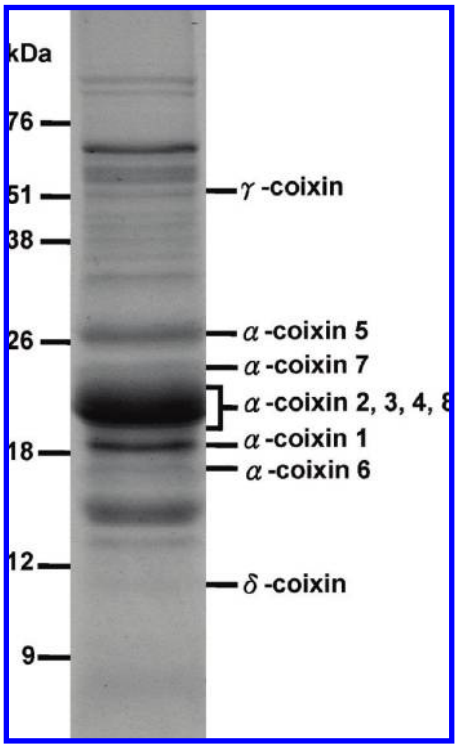

Figure 3. Identification of coixins in the protein extract of adlay grain resolved in SDS-PAGE. The crude extract of adlay grain was resolved in a 15% polyacrylamide gel under 120 V for 100 min. Protein bands corresponding to coixins were confirmed by LC-ESI-MS/MS analysis (**Table 2**).

lysine (on average 0.8, 0.6, and 0.1%, respectively); the δ-coixin is a sulfur-rich protein (18.2% methionine and 9.1% cysteine), and the γ-coixin is a nutritive protein composed of 2.0% methionine, 6.6% cysteine, and 2.6% lysine. Moreover, γ-coixin is also rich in histidine (8.9%), which is relatively low (ranging from 0 to 2.3%) in the other 9 coixins.

DISCUSSION

Taking advantage of EST sequencing and LC-ESI-MS/MS analysis in this study, we successfully identified 10 coixins, the most abundant storage proteins representing about 60% of the total proteins in adlay grain. An evaluation of the nutritional value of the 10 coixins indicates that the abundant α-coixins are low in methionine, cysteine, and lysine residues and that the well-balanced amino acid composition of adlay grain is putatively attributed to the occurrence of two nutritive proteins, δ-coixin and γ-coixin. Found in other cereal grains, homologous proteins of δ-coixin, for example, δ-prolamin, δ-kafirin, and δ-zein, are also sulfur-rich (**Figure 2B**); in contrast, those of γ-coixin, for example, γ-zein and γ-gliadins, are not nutritionally important (**Figure 2C**). It seems that γ-coixin is a unique nutritive protein in adlay grain.

Preparation of α-coixin was found to comprise four polypeptide classes of 27 kDa (C1), 25 kDa (C2), 17 kDa (C4), and 15 kDa (C5) in a previous study (*19*). The C1 and C2 protein bands, representing 80% of total *Coix* prolamins, corresponded to α-coixin 2, 3, 4, and 8 isoforms identified in the current study (**Figure 3**). Moreover, the C4 protein band corresponded to α-coixin 6 isoform, whereas the C5 protein band did not match any coixin isoform identified in the current study. Nevertheless, α-coixin 1, 5, and 7 isoforms found in this study did not match any of the four α-coixin classes reported previously. Possibly, α-coixin 1, 5, and 7 isoforms are present in the total

Table 2. Fragments of Coixins Identified by LC-ESI-MS/MS Analysis

coixin (MOWSE score)	residues	sequence
α-1 (221)	57−71	RLQQALAASILQQPL
	63−82	AASILQQPLAQLQQQSSAHL
	153−171	LQSQLFPCNPLVAANAAAY
	172−187	LQQQQLQQILPALSQL
	205−231	NQVAVANNAVYEQQHQLLQVNPLAAAF
	232−242	LQQQQRQLLPF
α-2 (369)	57−71	RLQQALAASILQQPL
	63−82	AASILQQPLAQLQQQSSAHL
	83−95	TIQTIAAQQQQQF
	154−172	LQSQLFPSNPLVAANAAAY
	217−232	EQQHQLLQVNPLAAAF
	233−243	LQQQQRQLLPF
	249−265	MNPALSWQQPIVGGVGF
α-3 (235)	57−71	RLQQALAASILQQPL
	63−82	AASILQQPLAQLQQQSSAHL
	83−95	TIQTIAAQQQQQF
	233−243	LQQQQRQLLPF
	249−265	MNPALSWQQPIVGGVGF
α-4 (85)	83−95	TIQTIAAQQQQQF
	250−266	MNPALSWQQPIVGGVGF
α-5 (138)	116−129	LASNPLAVANAVAY
	135−153	SQQFLPALSQLAVANPAAY
	160−172	SSNPLAAANTAAY
	179−188	QQILPALRQL
α-6 (268)	31−44	ASPTATIAQFLSPF
	58−80	RLQQTLAGSILQQPIAQLQQRSL
	89−111	AAQQNQELLPTLSQVAMLNPATY
	113−131	AGSILQQPIAQLQQRSL
	168−178	NQLDVAKAAAY
	179−197	LQQQQQLPINPLAVARLFL
α-7 (348)	65−75	QSLVVILRQPY
	76−87	SLLQQPSLANLF
	97−112	QQQLLPAINQVDAANL
	125−136	NQLARVNPAAYL
	144−168	NQQLAVASPIASLQQQLLPFYPQAL
	230−241	SQLALRNPTALL
	275−291	RNPATLLQQAIIGGAIF
α-8 (66)	54−64	RLQEAIAESIL
	79−86	QQLPLVNF
	226−242	RNPAASCQQPIVGAALF
δ (66)	83−99	AIGGSSLPTVVMQRQPF
	157−166	AQRPFPCCAF
γ (215)	206−217	LRQQCNPLVMPF
	218−231	LQSRLVQPSNCQVL
	232−252	RQQCCHELRQIEPQYLHQMIY
	303−314	HSCYPNNPYSSY

seed extract but not in the fraction of α-coixin in the previous study, or they may be separated from the abundant C1 and C2 protein bands under the electrophoretic condition of the current study.

Sequence analyses indicate that α-prolamins are sequentially composed of a hydrophobic signal sequence, an N-terminal tail region, several tandem repeats of approximately 20 residues, and a C-terminal tail; the variable lengths of α-prolamins mainly result from the number of tandem repeats in different isoforms (*17*). Being highly hydrophobic, α-prolamins tend to form oligomers that are packed into membrane-bound protein bodies (*20*). It has been proposed that the tandem repeats form a series of α-helices linked by glutamine-rich loops, and a three-dimensional model has been put forward in which a series of adjacent and antiparallel helices pack to form a distorted cylinder (*21*). Variable tandem repeats and some insertions are found in the eight α-coixins identified in this study (**Figure 2A**). Relative migration of these α-coixin isoforms resolved in SDS-PAGE is in agreement with their molecular masses (**Figure 3**).

Table 3. Deduced Amino Acid Compositions of Coixins

	amino acid composition (%)									
coixin	α-1	α-2	α-3	α-4	α-5	α-6	α-7	α-8	γ	δ
Met	0.82	0.82	0.82	0.82	1.37	0.57	0.37	0.45	1.97	18.18
Ala	16.87	16.80	17.21	16.33	19.18	16	13.28	14.48	1.97	9.09
Arg	1.23	1.23	1.23	0.82	1.37	1.71	2.21	2.26	1.97	2.80
Asn	5.76	5.33	5.74	5.71	5.82	4	4.43	5.43	2.95	1.40
Asp	0	0.41	0	0	0	0.57	0.74	0	0.66	0.70
Cys	0.82	0.41	0.41	0.41	0.68	0.57	0.37	1.36	6.56	9.09
Gln	22.22	21.72	22.54	22.04	21.92	19.43	22.14	20.81	31.15	13.29
Glu	0.82	0.82	0.41	0.41	0.34	1.71	0.74	1.36	5.25	0.70
Gly	1.65	1.64	1.64	1.63	0.68	1.14	0.74	0.45	4.60	4.20
His	1.65	1.64	1.23	1.63	1.03	2.29	0.37	0	8.85	1.40
Ile	4.53	4.51	4.51	4.90	4.11	5.14	4.43	5.43	1.64	2.10
Leu	15.23	15.57	15.57	16.73	16.44	16	17.71	18.10	4.59	5.59
Lys	0	0	0	0.41	0	0.57	0	0	2.62	0
Phe	3.70	4.10	3.69	3.67	3.42	4.57	5.9	5.88	0.98	2.80
Pro	8.64	8.61	8.61	8.16	7.53	8.57	8.49	9.05	7.54	7.69
Ser	5.76	5.74	6.56	6.12	6.51	4	5.17	7.24	5.90	9.09
Thr	1.23	1.64	1.23	1.63	1.71	4.57	3.32	1.36	2.62	4.90
Trp	0.41	0.41	0.41	0.41	0	0	0	0	0.33	0
Tyr	2.88	2.87	2.46	2.86	3.42	3.43	3.69	2.71	4.26	0.70
Val	5.76	5.74	5.74	5.31	4.45	5.14	5.90	3.62	3.61	6.29

Glutamine is the most abundant amino acid in the extracellular space of the human body and one of the few amino acids that directly crosses the blood-brain barrier (*22*, *23*). It is the most important donor of NH_3 in the kidney, playing an important role in the acid−base buffering system (*24*). Regarded as a conditionally essential amino acid, glutamine is supplemented when its metabolic demand exceeds the capacity of synthesis under certain conditions, such as major surgery, extensive burns, sepsis, and inflammation (*25*). Clinical trials in hospitalized adults and newborns have shown that glutamine supplementation decreases infectious complications, shortens hospital stays, improves baby growth, and decreases hospital costs in a number of patient populations (*26*−*28*). Amazingly, all 10 coixins are very rich in glutamine (**Table 3**). Accordingly, the adlay grain is rich in glutamine as observed in the chemical determination of its amino acid composition (*6*). The nutritional value of adlay grain may be partly attributed to its high level of glutamine in addition to its relatively rich protein content among cereal grains.

In light of the long-term consumption of adlay grain as a nourishing food source in Asian countries and its nutritive protein composition examined in this study, we suggest that adlay grain may be internationally utilized as a major high-quality food source. Nevertheless, several members in the prolamin superfamily, such as 2S albumins and nonspecific lipid transfer proteins, have been identified as allergens (*10*). Prolamins are resistant to thermal and proteolytic denaturation, presumably due to their highly stable structure, which may partly account for the allergenicity of the proteins (*29*). Sesame has been used as a food additive or key ingredient of diverse food products in Asian countries for many years, and thus sesame proteins do not seem to be allergens to most people in Asian countries. However, the incidence of sesame

allergy to people in Europe, Israel, the United States, and Australia has been increasingly reported in the past decade, possibly due to the spread of sesame products into new areas of the world and consequent exposure of new populations (*30*). The allergic factors in sesame are frequently ascribed to its sulfur-rich 2S albumin as well as other seed storage proteins (*31*). Similar to sesame, alday grain has been consumed locally in Asian countries and contains a sulfur-rich prolamin. Whether the sulfur-rich δ-coixin and other seed storage proteins in adlay grain are allergic factors to non-Asian people remains to be examined.

ABBREVIATIONS USED

EST, expressed sequence tag; LC-ESI-MS/MS, liquid chromatography−electrospray ionization−tandem mass spectrometry; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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