



Characterization of protein fractions from chickpea (*Cicer arietinum* L.) and oat (*Avena sativa* L.) seeds using proteomic techniques

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ABSTRACT

Albumin, globulin and glutelin fractions were prepared from chickpea and oat seeds using sequential extractions. Molecular characteristics of individual protein fractions were investigated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) in combination with proteomic techniques. SDS–PAGE results revealed that chickpea albumin and globulin fractions (C–Ab and C–Gb) showed protein bands with molecular weights (MWs) related to subunits of legumin (11S globulin) and pea vicilin (7S globulin); oat protein fractions (O–Ab, O–Gb and O–Gt) showed most protein bands with MWs related to subunits of oat 12S globulin (avenalin). With proteomic analysis, eighteen tryptic peptides from chickpea globulin fraction showed sequence homology that corresponded to chickpea legumin α - and β -subunit (NCBI accession number: gi|6273402; theoretical mass 56,216 Da) while sixteen tryptic peptides from chickpea albumin fraction (C–Ab) were identified as chickpea provicilin precursor (NCBI accession number: gi|82173888; theoretical mass 51,390 Da); fifteen tryptic peptides from oat protein fractions were identified with origin from oat 12S seed storage globulin 1 (NCBI accession number: gi|134918; theoretical mass 58,508 Da). The identified tryptic peptide, ALIVPQNFAIAAK, was commonly found in chickpea glutelin fraction (C–Gt), rice glutelin fraction (R–Gt), and oat albumin, globulin and glutelin fractions (O–Ab, O–Gb and O–Gt).

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1. Introduction

Proteomic techniques have been applied successfully for studying plant seeds over the past decade; examples including differentiation of the amphiphilic proteins from wheat kernel (Amiour, Merlino, Leroy, & Branlard, 2002), identification of the sesame (Beyer, Bardina, Grishina, & Sampson, 2002) and hazelnut allergen proteins (Beyer, Grishina, Bardina, Grishin, & Sampson, 2002), verification of the composition of soy protein for clinical studies (Gianazza, Eberini, Arnoldi, Wait, & Sirtori, 2003) and comparison of green coffee bean species (Gil-Agusti et al., 2005). Both chickpea (*Cicer arietinum* L.) and oats (*Avena sativa* L.) have relatively high protein contents (14.9–24.6% for chickpea, 12.4–24.5% for oat) and can be good sources of protein concentrates for food applications (Duke, 1981; Lászity, 1996). The storage proteins of chickpea seed have been fractionated into globulin (salt soluble; 56%), albumin (water soluble; 12%), prolamin (alcohol soluble; 2.8%), glutelin

(acid/alkali soluble; 18.1%), and residual proteins (Chavan, Kadam, & Salunkhe, 1989; Singh & Jambunathan, 1982). Oat groats possess the highest protein content (12.4–24.5%) among cereals (Lászity, 1996). Similar to chickpea protein, oat protein fractions are albumins (10–20%), globulins (12–55%), prolamins (12–14%) and glutelins (23–54%) (Haard, 1999).

Globulins represent about 50% of chickpea seed proteins and are composed of two major groups the 11S (legumin) and the 7S (vicilin) proteins (Casey, Domoney, & Smith, 1993). Unlike other cereals, oat is the only cereal that the predominant storage protein is soluble in salt solution and characterized as globulin. Oat globulins are a mixture of different proteins identified and separated as 12S (the major component), 7S and 3S fractions (Burgess, Shewry, Matlashewski, Altosaar, & Mifflin, 1983). Chickpea 11S (legumin) and oat 12S globulins are the major globulins; structurally, they are oligomeric proteins with six quaternary monomer 54–60 kDa subunits. The molecular weight (MW) of chickpea legumin is about 320–400 kDa (Casey et al., 1993) whereas the oat 12S globulin has a MW of 322 kDa (Peterson, 1978). Under reducing conditions with SDS–PAGE separation, the individual subunit, a monomer in native form, gives α - and β -subunits with MWs of about 32 and 22 kDa; these two subunits are connected by a single disulfide bond (Lászity, 1996; Sánchez-Vioque, Clemente, Vioque, Bautista, & Millán, 1999). Chickpea 7S (vicilin) globulin along with oat 3S and 7S globulins are storage proteins; their structure and conformation have been studied much less than 11S and 12S globulins. Guéguen (1991) reported

Abbreviations: AA(s), Amino acid(s); C–Ab, Chickpea albumin; C–Gb, Chickpea globulin; C–Gt, Chickpea glutelin; LC–ESI–MS/MS, Liquid chromatography electrospray ionization tandem mass spectrometry; MW(s), Molecular weight(s); m/z, Mass to charge ratio; NCBI, National Center for Biotechnology Information; O–Ab, Oat albumin; O–Gb, Oat globulin; O–Gt, Oat glutelin; R–Gt, Rice glutelin; SDS–PAGE, Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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subunit MWs of the pea 7S globulin as 50, 35, 33, 19, 15, and 13 kDa based on SDS-PAGE characterization, which are comparable to chickpea 7S vicilin subunits reported by Chang, Alli, Molina, Konishi, and Boye (2009). Oat 3S and 7S globulins are minor components of oat storage proteins; Burgess et al. (1983) characterized the major subunit of oat 7S globulin with MW of 55 kDa; some minor components of MW 65 kDa were also observed. Oat 3S globulin was found to have at least two major subunits with MWs of about 15 and 21 kDa (Burgess et al., 1983; Lásztity, 1996).

Chickpea is one of the few legumes containing a glutelin (cereal-like protein) whereas oat is unique among cereals with a relatively high amount of globulin (legume-like protein) as the major storage protein and a minor component of glutelins (Lásztity, 1996); the globulin proteins of both chickpea and oat have been studied by previous researchers (Kaur

& Singh, 2007; Robert, Nozzolillo, & Altosaar, 1985; Shotwell, Afonso, Davies, Chesnut, & Larkins, 1988; Singh & Jambunathan, 1982; Yust et al., 2003). Glutelins belong to the 11–12S globulin family; structurally glutelin is similar to globulin (Shotwell et al., 1988; Takaiwa, Ogawa, & Okita, 1999). However, there is little information on the molecular characterization of glutelins such as peptide sequences, amino acid sequences, biological properties and functional properties. The objectives of this study were to isolate protein fractions from chickpea and oat seeds and to characterize and compare these protein fractions using proteomic techniques including in-gel trypsin digestion, liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS), data analysis (MassLynx and ProteinLynx softwares) and on-line Mascot MS/MS ion search. Rice glutelin served as a reference protein to chickpea and oat glutelin fractions since it has been well characterized.

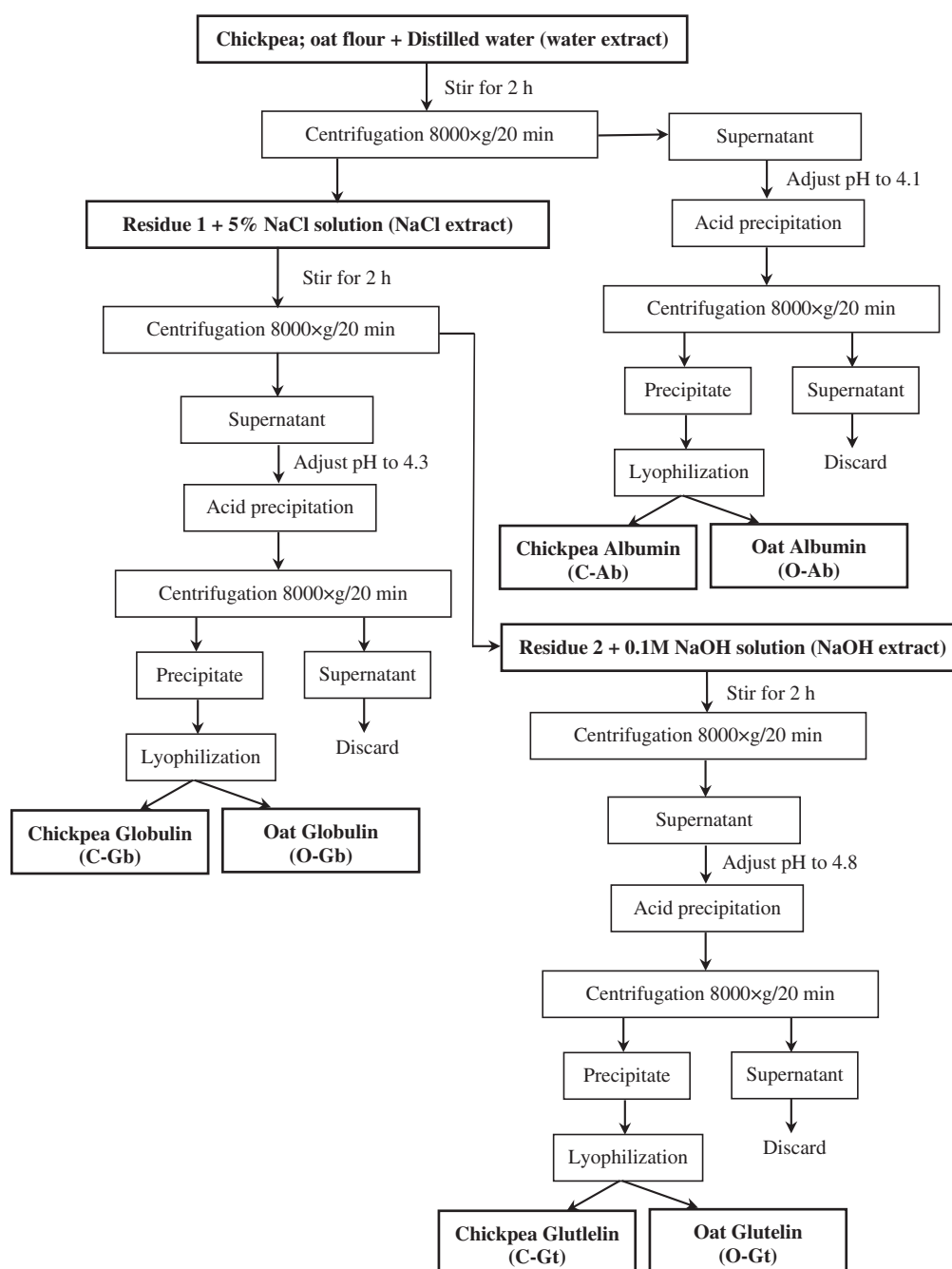


Fig. 1. Scheme of sequential protein extractions for chickpea and oat flour.

2. Materials and methods

2.1. Raw materials

Chickpea (Kabuli type) seeds and oat seeds were purchased from Emerson Milling Inc. (Emerson, MB, Canada), and rice grains (NuPak, USA) were purchased locally in Montreal; seeds were ground using a micro sample mill (Braun, KSM2 Type 4041, CA) and stored in air-tight plastic containers at room temperature until they were used.

2.2. Sequential extraction of proteins from chickpea and oat flour

Albumin, globulin, and glutelin fractions were extracted sequentially according to the procedure of Agboola, Ng, and Mills (2005) with modifications (Fig. 1). A sample (50 g) of chickpea or oat flour was

mixed with distilled water (200 mL). The mixture was allowed to stand with intermittent stirring for 2 h then centrifuged ($8000\times g$, 20 min, Beckman, Model JA2-21, USA). The supernatant was filtered with fine glass wool then precipitated by addition of 1 M HCl to pH 4.1 with subsequent centrifugation ($8000\times g$, 20 min); this albumin fraction (chickpea albumin (C-Ab); oat albumin (O-Ab)) was obtained after lyophilization of the precipitate and the supernatant was discarded. The residue 1 was mixed with 5% NaCl (200 mL) and stirred for 2 h then centrifuged ($8000\times g$, 20 min); the supernatant was filtered with fine glass wool and then precipitated by addition of 1 M HCl to pH 4.3 with subsequent centrifugation ($8000\times g$, 20 min); this globulin fraction (chickpea globulin (C-Gb); oat globulin (O-Gb)) was recovered after lyophilization of the precipitate and the supernatant was discarded. The residue 2 was mixed with 0.1 M NaOH (200 mL) and stirred for 2 h then centrifuged ($8000\times g$, 20 min); the supernatant was filtered with fine

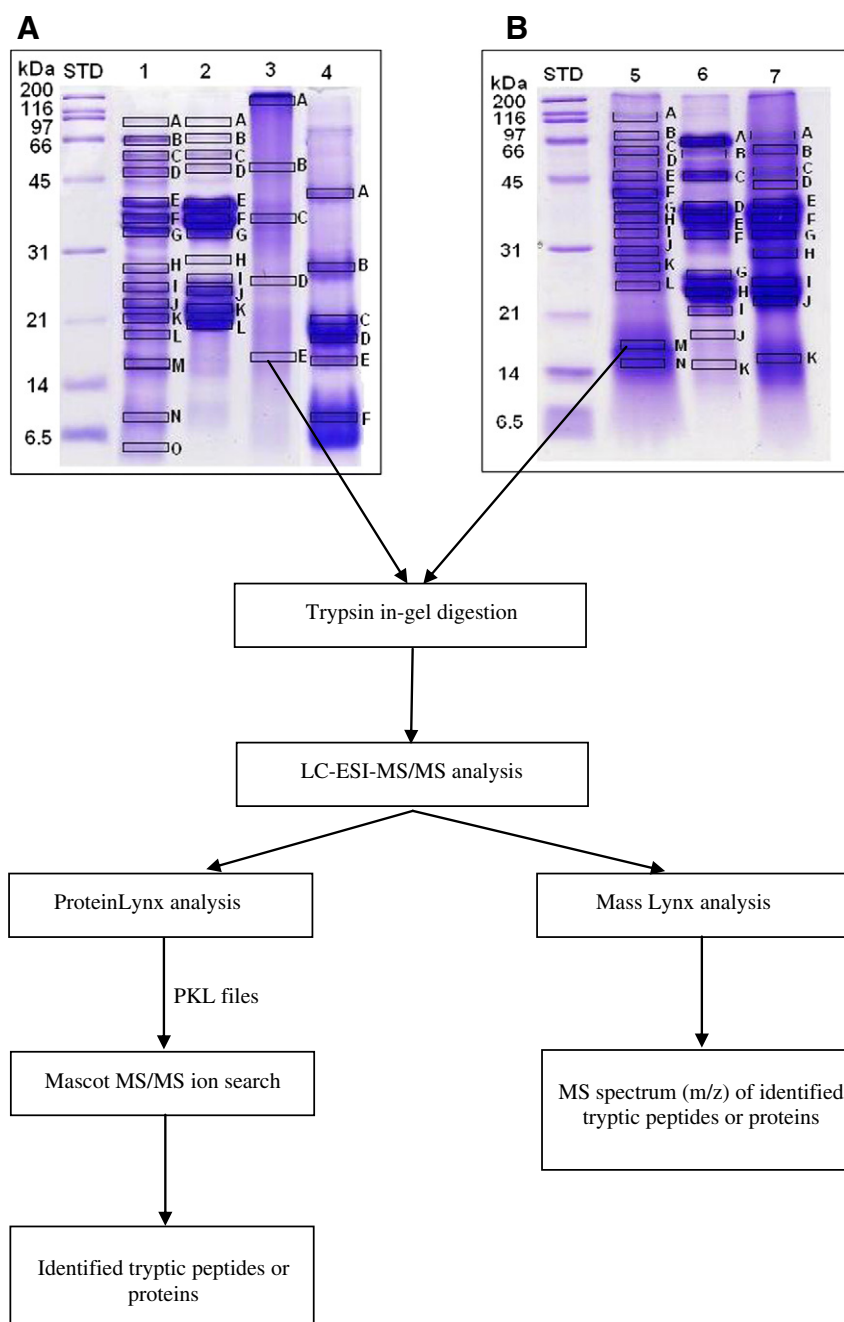


Fig. 2. Experimental proteomic approach for identifying partial tryptic peptide sequences of chickpea, oat and rice protein fractions.

glass wool and then precipitated by addition of 1 M HCl to pH 4.8 with subsequent centrifugation (8000×g, 20 min); this glutelin fraction (chickpea glutelin (C-Gt); oat glutelin (O-Gt)) was recovered after lyophilization of the precipitate and the supernatant was discarded. All the freeze-dried protein isolates were stored at -20°C until further use.

2.3. Extraction of rice glutelin

Rice glutelin fraction (R-Gt) was prepared by extraction using 0.02% NaOH followed by acid precipitation (1 M HCl to pH 4.8); the precipitate was lyophilized and designated as rice glutelin fraction (Chandna & Matta, 1990); it was used as a reference protein since there is little information on glutelins in chickpea and oat.

2.4. Determination of total soluble protein content

The total soluble protein contents of chickpea, oat and rice protein isolates were determined by a modified Lowry assay (Bensadoun & Weinstein, 1976). A standard curve was prepared using 2–100 μg of bovine serum albumin (BSA). All analyses were conducted in triplicate. The total soluble protein contents of C-Ab, C-Gb, C-Gt, R-Gt, O-Ab, O-Gb, and O-Gt were determined as 43.0, 93.8, 27.5, 67.4, 65.7, and 77.2%, respectively.

2.5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using the technique described by Laemmli (1970) with a 4% stacking gel and a 12% resolution gel. Sample solutions (20 μL) were prepared from 10 mg freeze-dried protein extract or precipitates dissolved in 1 ml sample buffer (distilled water, 0.5 M Tris-HCl pH 6.8, glycerol, 10% SDS, 1% bromophenol blue and β -mercaptoethanol) heated at 98°C for 10 min, then applied to the sample wells. The standard protein marker (broad range molecular weight, Bio-Rad Hercules, USA) which contained myosin (200,000 Da), β -galactosidase (116,250 Da), phosphorylase b (97,400 Da), serum albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), trypsin inhibitor (21,500 Da), lysozyme (14,400 Da) and aprotinin (6,500 Da), was used to prepare a standard curve for molecular weight estimation. Electrophoretic migration was monitored at constant current (14 mA/gel) for 1.5 to 2 h. Gels were fixed with fixing solution (water/methanol/acetic acid/700 ml:200 ml:100 ml) for 30 min and then stained with Coomassie Brilliant Blue R-250 for 1 h. The stained

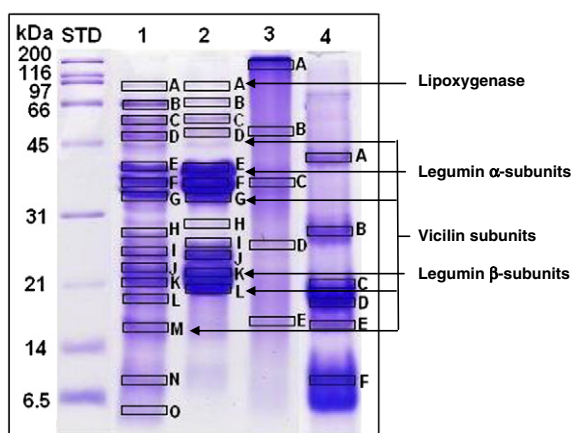


Fig. 3. Twelve percent SDS-PAGE of chickpea protein isolates from sequential extractions. STD: Standard protein markers; (1) C-Ab fraction; (2) C-Gb fraction; (3) C-Gt fraction; (4) R-Gt fraction.

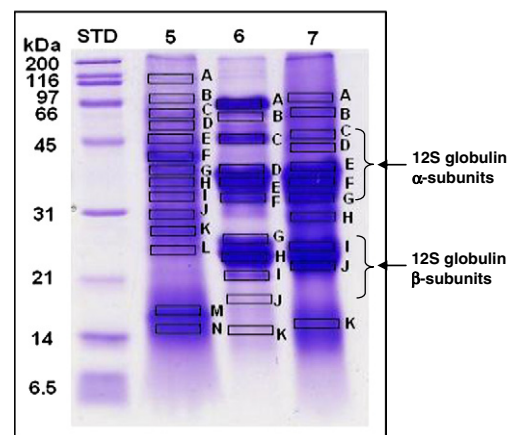


Fig. 4. Twelve percent SDS-PAGE of oat protein isolates from sequential extractions. STD: Standard protein markers; (5) O-Ab fraction; (6) O-Gb fraction; (7) O-Gt fraction.

gels were destained by frequently changing the fixing solution until the excess stain disappeared.

2.6. Proteomic techniques

Fig. 2 shows the schematic workflow of the proteomic techniques used to characterize the protein fractions and to identify tryptic peptide fragments from chickpea, oat and rice protein fractions prepared previously; separated protein bands (1A–1O from C-Abfraction; 2A–2L from C-Gb fraction; 3A–3E from C-Gt fraction; 4A–4F from R-Gt fraction; 5A–5N from O-Abfraction; 6A–6K from O-Gb fraction; 7A–7K from O-Gt fraction) from 12% SDS-PAGE gels (Fig. 3 for chickpea and rice protein fractions and Fig. 4 for oat protein fractions) were excised, destained and digested with trypsin enzyme; subsequently the tryptic peptide fragments were analyzed and identified by LC–ESI–MS/MS, ProteinLynx and MassLynx analysis, and Mascot MS/MS ion search.

2.6.1. Gel destain and in-gel tryptic digestion

The gel destain and in-gel trypsin digestion protocols were modified from the methods reported by Rosenfeld, Capdevielle, Guillemot, and Ferrara (1992) and Shevchenko, Wilm, Vorm, and Mann (1996). Selected protein bands of one dimension (1D) SDS-PAGE gels (Figs. 3 and 4) from chickpea, rice and oat protein fractions were excised manually using 250 μL pipette tips then transferred to sterilized Eppendorf tubes (1.5 mL). The gel pieces were destained twice with 200 μL 100 mM NH_4HCO_3 /50% acetonitrile for 2×45 min at 37°C . To break disulfide linkages in the proteins, the gel pieces were treated with 50 μL 10 mM DTT in 50 mM NH_4HCO_3 and incubated for 30 min at 56°C and centrifuged (13,000 rpm, 5 min). After removal of supernatant, the gel pieces were alkylated with 100 μL 55 mM iodoacetamide in 50 mM NH_4HCO_3 and incubated for 10 min at room temperature. The gel pieces then dehydrated and rehydrated with 100 μL 100% CH_3CN and 50 μL 50 mM NH_4HCO_3 ; the dehydration step was repeated with CH_3CN and the gel pieces were air-dried at room temperature. For trypsin (modified porcine trypsin, sequencing grade, Promega, Madison, WI) digestion, 30 μL of trypsin working solution (20 μg trypsin/3 mL 50 mM NH_4HCO_3) was added to each sample including a blank sample (with non-protein gel pieces from SDS-PAGE) as a negative control and samples were incubated for 4 h at 37°C .

After incubation, the samples were centrifuged (13,000 rpm, 1 min) and the supernatants were transferred to Eppendorf tubes; 30 μL of extraction buffer (90% CH_3CN /0.5 M Urea) was added to the remaining gel pieces and supernatants were recovered by centrifugation (13,000 rpm, 1 min). The supernatants were combined with the respective original supernatants. This gel extraction procedure was repeated twice. The

Protein	MWs reported by previous researchers (KDa)	MWs of chickpea and rice protein fractions obtained in present work (Fig. 3)				Protein	MWs reported by previous researchers (KDa)	MWs of oat protein fractions obtained in present work (Fig. 4)		
		C-Ab	C-Gb	C-Gt	R-Gt			O-Ab	O-Gb	O-Gt
Lipoxygenase	92 ¹	93.6 (1A)	93.6 (2A)			12S	50–70 ⁷	69.6 (5B),	65.8 (6A),	69.3 (7A),
Legumin (11S)	46.5–39.8 ² (α-subunit) 24.3–25.3 ² (β-subunit)	41.7 (1E), 38.9 (1F), 25.9 (1I), 24.3 (1J)	40.7 (2E), 38.0 (2F), 26.5 (2I), 25.2 (2J)			globulin α-subunit	32–43 ⁷	62.3, (5C), 45.7 (5E), 43.2 (5F), 39.3 (5G), 36.9 (5H), 33.7 (5I)	60.9 (6B), 45.9 (6C), 39.8 (6D), 36.8 (6E), 33.2 (6F)	62.5 (7B), 46.2 (7C), 44.8 (7D), 36.7 (7F), 33.5 (7G),
Pea vicilin (7S)	50 ³ 35 ³ 33 ³	51.0 (1D) 36.3 (1G)	51.0 (2D) 34.6 (2G)			β-subunit	19–25 ⁷	25.8 (5L), 17.2 (5M)	24.1 (6H), 22.0 (6I), 18.5 (6J)	26.2 (7I), 23.5 (7J)
	19 ³ 15 ³	19.0 (1L) 16.5 (1M)	19.2 (2L)			7S globulin	65 ⁸			
							55 ⁸	55.2 (5D)		
						3S globulin	21 ⁸			
Rice glutelin	13 ³ 55 ⁴ 38.5 ⁵ 21.4 ⁵	55.3 (1C) 21.0 (1K)	56.0 (2C) 22.4 (2K)	51.0 (3B) 38.1 (3C)	48.6 (4A)		15 ⁸	15.0 (5N)	15.1 (6K)	41.5 (7E)
					21.5 (4C), 19.0 (4D)					
	15.8 ⁵			17.1 (3E)	16.3 (4E)					16.1 (7K)
Albumin (2S)	12 ⁶ , 10 ⁶	8.0 (1N)								
Unknown		63.6 (1B), 28.5 (1H), 5.3 (1O)	63.6 (2B), 29.2 (2H)	200 (3A), 26.2 (3D)	8.0 (4F), 28.6 (4B)			98.2 (5A), 30.9 (5J), 28.5 (5K)	27.8 (6G)	30.7 (7H)

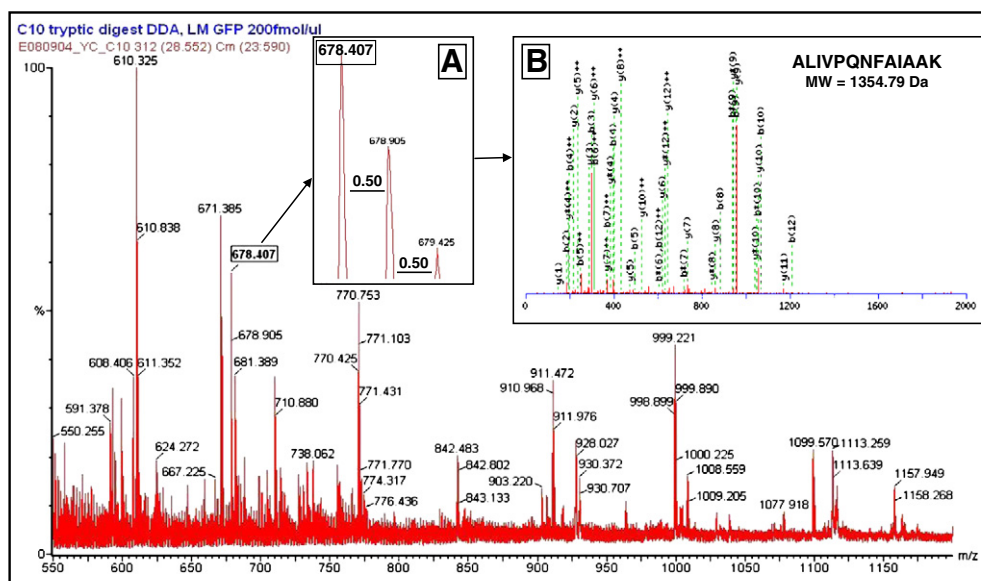


Fig. 5. LC-ESI-MS spectra (m/z region 550 to 1200) of chickpea protein band 1J, m/z 678.407 signal in bold type. Insert A shows the identified doubly charged signal by the difference of 0.50 between signals. Insert B represents the fragmentation of LC-ESI-MS/MS spectra of the peptide ALIVPQNFAIAAK, calculated MW 1354.79 Da.

comparable to the chickpea lipoxygenase (92 kDa) (Clemente et al., 2000); protein bands 1E, 1F, 1I, 1J, 2E, 2F, 2I, and 2J are likely the legumin α - and β -subunits (Chang et al., 2009; Sánchez-Vioque et al., 1999); protein bands 1D, 1G, 1L, 1M, 2D, 2G, and 2L are comparable to 7S pea vicilin subunits (Guéguen, 1991); protein bands 1C, 1K, 2C, and 2K showed similar MWs to rice glutelin subunits (Agboola et al., 2005; Takaiwa et al., 1999); protein band 1N with MW 8.0 kDa could be a subunit from 2S albumin (Vioque et al., 1999). The SDS-PAGE electrophoretic patterns of C-Ab and C-Gb fractions agree with other studies on chickpea albumin and globulin fractions (Kaur & Singh, 2007; Liu, Hung, & Bennett, 2008). In lane 3, most of the observed protein bands (3B, 3C, and 3E) from fraction C-Gt are comparable to rice glutelin subunits (Agboola et al., 2005; Takaiwa et al., 1999). Protein bands of C-Gt fraction were different from protein bands of R-Gt fraction as shown in lane 4 (Fig. 3); this could be the R-Gt fraction was extracted without removing the rice albumin and globulin fractions and resulted in different protein bands.

For oat protein fractions (Fig. 4), 14 protein bands were observed from O-Ab fraction (lane 5) and the MWs of protein bands were 98.2 (5A), 69.6 (5B), 62.3 (5C), 55.2 (5D), 45.7 (5E), 43.2 (5F), 39.3 (5G), 36.9 (5H), 33.7 (5I), 30.9 (5J), 28.5 (5K), 25.8 (5L), 17.2 (5M) and 15 (5N) kDa; 11 protein bands were observed from O-Gb fraction (lane 6) and the MWs of protein bands were 65.8 (6A), 60.9 (6B), 45.9 (6C), 39.8 (6D), 36.8 (6E), 33.2 (6F), 27.8 (6G), 24.1 (6H), 22.0 (6I), 18.5 (6J) and 15.1 (6K) kDa; 11 protein bands were observed from O-Gt fraction (lane 7) and the MWs of protein bands were 69.3 (7A), 62.5 (7B), 46.2 (7C), 44.8 (7D), 41.5 (7E), 36.7 (7F), 33.5 (7G), 30.7 (7H), 26.2 (7I), 23.5 (7J), and 16.1 (7K) kDa. Lásztity (1996) reported that the oat 12S globulin monomers with MW ranging from 50 to 70 kDa and the comparable protein bands (5B, 5C, 6A, 6B, 7A and 7B) were observed in all three oat protein fractions as 12S globulin; protein bands 5E, 5F, 5G, 5H, 5I, 6C, 6D, 6E, 6F, 7C, 7D, 7F and 7G were comparable to the α -subunit (32–43 kDa) from oat 12S globulin along with the protein bands 5L, 5M, 6H, 6I, 6J, 7I and 7J were comparable to

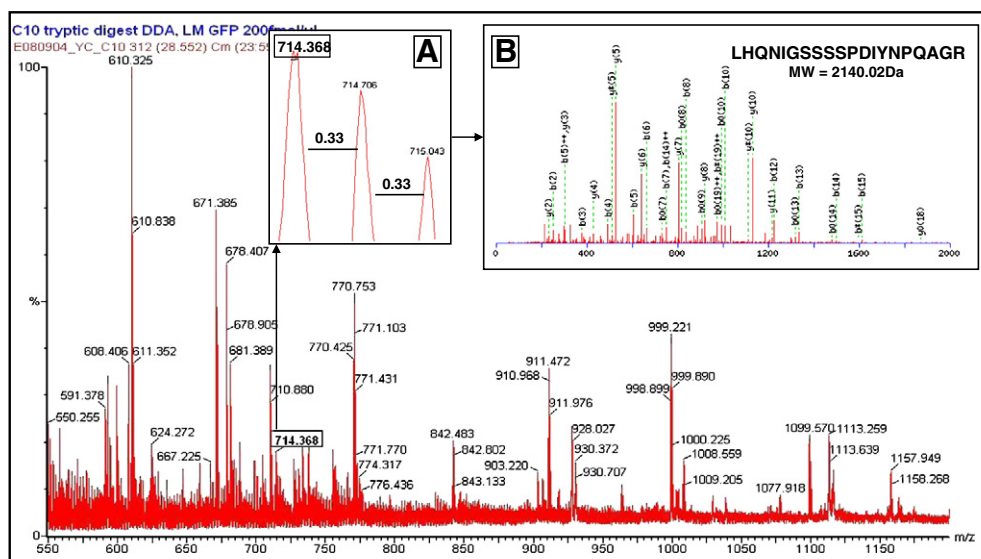


Fig. 6. LC-ESI-MS spectra (m/z region 550 to 1200) of chickpea protein band 1J, m/z 714.368 signal in bold type. Insert A shows the identified triply charged signal by the difference of 0.33 between signals. Insert B represents the fragmentation of LC-ESI-MS/MS spectra of the peptide LHQNISSSSPDYINPQAGR, calculated MW 2140.02 Da.

Table 2

Identified tryptic peptides of SDS-PAGE protein bands (Fig. 3) from C-Ab (lane 1), C-Gb (lane 2) and C-Gt (lane 3) fractions with origin from chickpea legumin α - and β -subunit [*Cicer arietinum*] (NCBI accession number: gil6273402; theoretical mass 56,216 Da).

Peptide sequences ^a	MW (Da)	Identified in SDS-PAGE protein bands (Fig. 3)
Legumin α-subunit^b		
IKSEGLIETWNPNSK _{45–60}	1771.91	1C (55.3 kDa), 2C (56.0 kDa), 2F (38.0 kDa), 2G (34.6 kDa)
SEGLIETWNPNSK _{47–60}	1530.73	1F (38.9 kDa), 2B (63.6 kDa), 2C (56.0 kDa), 2E (40.7 kDa), 2F (38.0 kDa), 2G (34.6 kDa), 2J (25.2 kDa), 2L (19.2 kDa), 3C (38.1 kDa)
RFYLAGNHEQEFLR _{180–193}	1778.88	1F (38.9 kDa), 2C (56.0 kDa), 2F (38.0 kDa), 2G (34.6 kDa), 2L (19.8 kDa), 3C (38.1 kDa)
FYLAGNHEQEFLR _{181–193}	1622.78	1C (55.3 kDa), 1F (38.9 kDa), 2C (56.0 kDa), 2E (40.7 kDa), 2G (34.6 kDa), 3B (51.0 kDa)
YQEGSEEEENEGGNIFSGFK _{194–214}	2376.99	1C (55.3 kDa), 1F (38.9 kDa), 2E (40.7 kDa), 2F (38.0 kDa), 2G (34.6 kDa), 3C (38.1 kDa)
YQEGSEEEENEGGNIFSGFKR _{194–215}	2533.09	2B (63.6 kDa), 2C (56.0 kDa), 2E (40.7 kDa), 2G (34.6 kDa)
RDFLEDALNVNR _{215–227}	1460.73	1E (41.7 kDa)
DFLEDALNVNR _{216–226}	1304.63	1C (55.3 kDa), 1F (38.9 kDa), 1K (21.0 kDa), 2C (56.0 kDa), 2E (40.7 kDa), 2F (38.0 kDa), 2G (34.6 kDa), 2H (29.2 kDa), 2I (25.2 kDa), 3A (200 kDa), 3B (51.0 kDa), 3C (38.1 kDa)
DFLEDALNVNRR _{216–227}	1460.73	1E (41.7 kDa), 1F (38.9 kDa), 2B (63.6 kDa), 2C (56.0 kDa), 2E (40.7 kDa), 2F (38.0 kDa), 2G (34.6 kDa), 3B (51.0 kDa), 3C (38.1 kDa)
NEDEEKGAIVK _{236–246}	1230.60	1C (55.3 kDa), 1E (41.7 kDa), 1F (38.9 kDa), 2C (56.0 kDa), 2E (40.7 kDa), 2F (38.0 kDa), 2G (34.6 kDa), 3C (38.1 kDa)
Legumin β-subunit^c		
LHQNIGSSSPDIYNPQAGR _{322–341}	2140.02	1C (55.3 kDa), 1E (41.7 kDa), 1J (24.3 kDa), 1K (21.0 kDa), 2B (63.6 kDa), 2C (56.0 kDa), 2E (40.7 kDa), 2G (34.6 kDa), 2H (29.2 kDa), 2I (26.5 kDa), 2J (25.2 kDa), 2K (22.4 kDa), 3A (200 kDa), 3B (51.0 kDa), 3C (38.1 kDa)
FLKLSAEFGSLHK _{355–367}	1475.81	1J (24.3 kDa), 1K (21.0 kDa), 2C (56.0 kDa), 2K (22.4 kDa), 3A (200 kDa), 3E (17.1 kDa)
LSAEFGSLHK _{358–367}	1087.56	1C (55.3 kDa), 2J (25.2 kDa), 2K (22.4 kDa), 2L (19.8 kDa), 3A (200 kDa), 3C (38.1 kDa)
NAMFVPHYNLNANSILYALK _{368–387}	2308.16	1J (24.3 kDa), 1K (21.0 kDa)
NAMFVPHYNLNANSILYALKGR _{368–389}	2521.28	1J (24.3 kDa), 1K (21.0 kDa)
GNSVFDGELEAGR _{400–412}	1349.62	1K (21.0 kDa), 2I (26.5 kDa), 2L (19.8 kDa), 3A (200 kDa), 3E (17.1 kDa)
ALIVPQNFAIAAK _{413–425}	1354.79	1J (24.3 kDa), 1K (21.0 kDa), 2C (56.0 kDa), 2J (25.2 kDa), 2K (22.4 kDa), 3B (51.0 kDa), 3C (38.1 kDa)
SLSDRFSYVAFK _{426–437}	1418.71	1J (24.3 kDa), 1K (21.0 kDa), 2B (63.6 kDa), 2I (26.5 kDa), 2J (25.2 kDa), 2K (22.4 kDa), 3A (200 kDa), 3E (17.1 kDa)
FSYVAFK _{431–437}	860.44	1J (24.3 kDa), 1K (21.0 kDa), 2C (56.0 kDa), 2J (25.2 kDa), 2K (22.4 kDa), 2L (19.8 kDa), 3B (51.0 kDa), 3C (38.1 kDa)

^a Amino acids are presented in single letter code within peptide sequences.

^b Legumin α -subunit: AAs position located between 22 and 311.

^c Legumin β -subunit: AAs position located between 312 and 496.

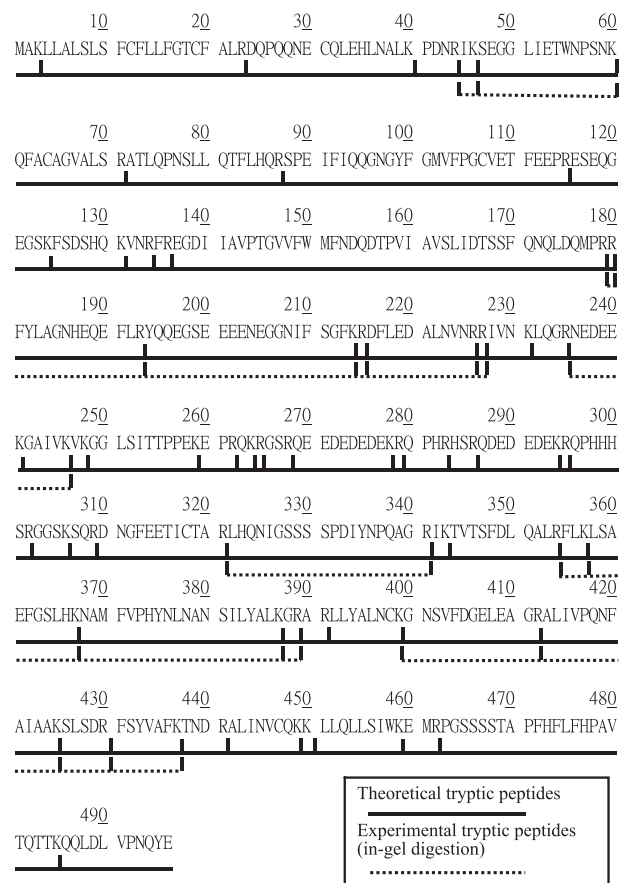


Fig. 7. Amino acids sequence of identified tryptic peptides with origin from chickpea legumin α - and β -subunit [*Cicer arietinum*] (NCBI accession number: gil6273402; theoretical mass 56,216 Da).

β -subunits (19–25 kDa) from oat 12S globulin reported by the same author. Furthermore O-Ab and O-Gb fractions showed protein bands 5D, and 5N and 6K that were in relation to 7S (55 kDa) and 3S (15 kDa) globulin, respectively (Burgess et al., 1983); the O-Gt fraction showed similar protein bands (7E and 7K) to rice glutelin subunits (38.5 and 15.8 kDa) (Agboola et al., 2005).

3.2. Identified tryptic peptide sequences with origin from chickpea legumin and chickpea provicilin precursor

The results from Mascot MS/MS ion search for the in-gel tryptic digests of each protein bands from chickpea, rice and oat protein fractions, revealed that all the identified tryptic peptides from chickpea, rice and oat protein fractions were listed as doubly or triply charged peptides.

Figs. 5 and 6 show two representative examples of doubly and triply charged peptides identified from proteomic analysis. Fig. 5 shows the identification of the doubly charged peptide, ALIVPQNFAIAAK (MW 1354.79 Da) listed from Mascot MS/MS ion search with origin from chickpea legumin, from protein band 1J (C-Ab fraction; lane 1, Fig. 3); the observed signal with m/z 678.407 was doubly charged (difference of 0.5 between adjacent signals as shown in the insert A, the insert B represents the final result of the identified peptide from the LC-ESI-MS/MS spectra). Similarly, Fig. 6 shows the identification of a triply charged peptide LHQNIGSSSPDIYNPQAGR (MW 2140.02 Da) from the same protein band (1J); the observed signal with m/z 714.368 was triply charged (difference of 0.33 between adjacent signals as shown in the insert A, the insert B represents the final result of identified peptide from LC-ESI-MS/MS spectra).

Table 2 summarizes the identified tryptic peptides of protein bands (Fig. 3) from C-Ab (lane 1), C-Gb (lane 2) and C-Gt (lane 3) fractions from chickpea legumin α - and β -subunit [*Cicer arietinum*] (NCBI accession number: gi|6273402; theoretical mass 56,216 Da). The chickpea legumin (496 amino acids) is composed of a signal peptide at the amino acid (AA) position 1–21, an acidic polypeptide chain (α -subunit) at AA position 22–311, and a basic polypeptide chain (β -subunit) at AA position 312–496; a disulfide bond is observed at AA position 31–64 and an interchain (between α - and β -chain) is observed at AA position 107–318 (Mandaokar & Koundal, 1997). Table 2 shows 10 tryptic peptides from chickpea albumin, globulin and glutelin fractions (C-Ab, C-Gb and C-Gt, respectively) which were identified as legumin α -subunit and 9 were identified as legumin β -subunit; most of the identified tryptic peptides with origin from chickpea legumin were present in all chickpea protein fractions except peptides, RDFLEDALNVNR, NAMFVPHYNLNAN-SILYALK and NAMFVPHYNLNANSILYALKGR which were found in chickpea albumin fraction (C-Ab; lane 1, Fig. 3). Protein bands 1C (55.3 kDa), 2B (63.6 kDa), 2C (56.0 kDa) and 3B (51.0 kDa) showed tryptic peptides present in both legumin α - and β -subunits; this suggests these protein bands can be the legumin parent (monomer) protein consisting of α -subunit and β -subunit (Mandaokar & Koundal, 1997). Protein bands 1F (38.9 kDa), 2F (38.0 kDa), 2G (34.6 kDa) showed tryptic peptides mostly from the legumin α -subunit; protein bands 1J (24.3 kDa), 1K (21.0 kDa), 2J (25.2 kDa), 2K (22.4 kDa) and 3E (17.1 kDa) showed tryptic peptides mostly from the legumin β -subunit; this suggests these protein bands (1F, 2F and 2G; 1J, 1K, 2J, 2K and 3E) can be the legumin α - and β -subunits, respectively; SDS-PAGE MWs of protein bands (1F, 2F; 2J) corresponded to MWs of legumin α - and β -subunits, respectively, reported by Sánchez-Vioque et al. (1999). Protein bands 1E (41.7 kDa), 2E (40.7 kDa), 2H (29.2 kDa), 2I (26.5 kDa), 2L (19.8 kDa) and 3C (38.1 kDa) showed tryptic peptides from either the legumin α - or β -subunits.

Fig. 7 shows the AAs sequence of chickpea legumin with the tryptic peptides from chickpea protein fractions (C-Ab, C-Gb and C-Gt) identified during the proteomic analysis; the solid line represents the theoretical peptides by trypsin cleavage and the broken line represents

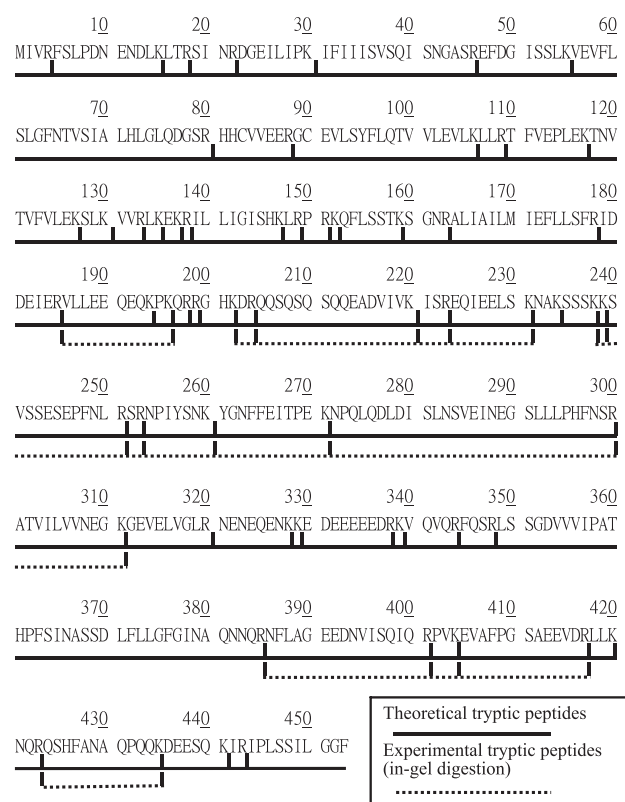


Fig. 8. Amino acids sequence of identified tryptic peptides with origin from chickpea provicilin precursor [*Cicer arietinum*] (NCBI accession number: gi|82173888; theoretical mass 51,390 Da).

the experimentally obtained tryptic peptides identified from in-gel digestion. Theoretically, 59 cleavage sites at either arginine (R) or lysine (K) of legumin AAs sequence are available for tryptic hydrolysis; two cleavage sites at AAs position 40–41 (KP) and 462–463 (RP) are not available since trypsin does not react to proline at C-terminal; 25 cleavage sites appeared in identified tryptic peptides derived from in-gel digestion. Based on the AAs sequence, 155 AAs from identified tryptic peptides out of 496 total AAs contributes to 31% sequence coverage.

Tryptic peptides identified (listed in Table 3) with origin from chickpea provicilin precursor [*Cicer arietinum*] (NCBI accession number: gi|82173888; theoretical mass 51,390 Da) were only found in protein bands 1G (36.3 kDa), 1H (28.5 kDa) and 1M (16.5 kDa) from C-Ab fraction; two peptides sequences, EQIEELSK_{224–231} and SRNPIYSNK_{252–260}, appeared frequently in protein bands 1G, 1H and 1M. This suggests that the chickpea 7S vicilin fractions could be partially extracted during the water extraction process. Fig. 8 presents the AAs sequence of chickpea provicilin precursor with the identified tryptic peptides from C-Ab fraction; the solid line and the broken line represents the theoretical and experimental (in-gel digestion) tryptic peptides, respectively. In theory, 60 cleavage sites at either arginine (R) or lysine (K) of chickpea provicilin precursor AAs sequence are available for tryptic hydrolysis; one cleavage site at AAs position 149–150 (RP) is not available due to trypsin does not react to proline at C-terminal; 21 cleavage sites appeared in identified tryptic peptides derived from in-gel digestion. 157 AAs are accountable from identified tryptic peptides; it gives 34% sequence coverage out of 453 total AAs from chickpea provicilin precursor.

3.3. Identified tryptic peptide sequences with origin from rice proteins

Table 4 summarizes the tryptic peptides of protein bands from C-Gt and R-Gt fractions corresponding to rice proteins. The protein band 3D (26.2 kDa) from the C-Gt fraction showed tryptic peptide sequence,

Table 3

Identified tryptic peptides of SDS-PAGE protein bands (Fig. 3) from chickpea albumin (lane 1) fraction with origin from provicilin precursor [*Cicer arietinum*] (NCBI accession number: gi|82173888; theoretical mass 51,390 Da).

Peptide sequences ^a	MW (Da)	Identified in SDS-PAGE protein bands (Fig. 3)
VLLEEQEQPK _{186–196}	1339.73	1H (28.5 kDa)
DRQQSQSQSQEQADVIVK _{203–220}	2073.00	1G (36.3 kDa)
QQSQSQSQSQEQADVIVK _{205–220}	1801.88	1G (36.3 kDa), 1H (28.5 kDa)
ISREQEELSK _{221–231}	1330.70	1G (36.3 kDa), 1H (28.5 kDa)
EQIEELSK _{224–231}	974.49	1G (36.3 kDa), 1H (28.5 kDa), 1M (16.5 kDa)
KSVSSESEPFNL _{239–251}	1478.73	1G (36.3 kDa), 1H (28.5 kDa)
SVSSESEPFNL _{240–251}	1350.64	1M (16.5 kDa), 1H (28.5 kDa)
SRNPIYSNK _{252–260}	1077.55	1G (36.3 kDa), 1H (28.5 kDa), 1M (16.5 kDa)
NPIYSNK _{254–260}	834.42	1G (36.3 kDa)
YGNFFEITPEKNPQLQDL _{272–300}	4587.29	1H (28.5 kDa)
ISLNSVEINEGSLLLPHFNSR _{261–300}		
NPQLQDLDSLNSV _{272–300}	3261.66	1G (36.3 kDa), 1H (28.5 kDa)
ATVILVNEGK _{301–311}	1141.67	1M (16.5 kDa)
NFLAGEEDNVISQIQRPVK _{386–404}	2156.12	1M (16.5 kDa)
EVAFPGSAEEVD _{405–417}	1404.65	1M (16.5 kDa)
QSHFANAQPOQK _{424–435}	1382.66	1M (16.5 kDa)

^a Amino acids are presented in single letter code within peptide sequences.

Table 4

Summary of tryptic peptides identified from C-Gt and R-Gt fractions corresponding to rice proteins.

Protein bands excised from SDS-PAGE (Fig. 3)	Identified peptide sequences ^a	MW (Da)	Sequence coverage (%)	Protein origin	Accession number from NCBI database	Theoretical mass (Da)	Mascot score
<i>Fraction C-Gt^b</i>							
3D (26.2 kDa)	ALPVDVIANAYR _{429–440}	1300.71	2	Glutelin C precursor [<i>Oryza sativa</i> (japonica cultivar-group)]	gi 37993736	54855	59
<i>Fraction R-Gt^c</i>							
4A (48.6 kDa)	LQAFEPLR _{50–58}	972.53	15	Glutelin precursor [<i>Oryza sativa</i> (japonica cultivar-group)]	gi 62546207	56383	241
4B (28.6 kDa)	VIQPGQLLVPR _{87–97}	1218.74	33	Glutelin [<i>Oryza sativa</i> (japonica cultivar-group)]	gi 31455453	36038	634
	QKEFLAGNNNR _{196–207}	1402.73					
	ISSVNSQKFPILNLIQMSATR _{329–349}	2362.26					
	TNANAFVSHLAGK _{426–437}	1328.68					
	ALPVDVIANAYR _{442–453}	1286.69					
	FDRLQAFEPLR _{47–57}	1390.73					
	VIEPQGLLVPR _{86–97}	1219.72					
	RVIEPQGLLVPR _{87–97}	1375.83					
	YSNTPGMVYIIQGR _{98–111}	1613.78					
	DEHQKIHQFR _{145–154}	1336.66					
4C (21.5 kDa)	QKEFLAGNNNR _{196–207}	1402.73	36	19 kDa globulin precursor [<i>Oryza sativa</i> (japonica cultivar-group)]	gi 20159	21492	350
	SIEQHSGQNIFSGFNELLSEAL	3285.66					
	GVNALVAK _{215–245}	1525.79					
	LQGQNDQGEIIR _{247–259}	1288.57					
	EVQDSPLDACR _{39–49}	1288.57					
	EVQDSPLDACRQVLDR _{39–54}	1899.91					
	FQPMFR _{62–67}	840.39					
	RPGALGLR _{68–75}	838.51					
	MQCCQLQDVSR _{76–87}	1551.65					
	QYAAQLPSMCRVEPQQCSIFAAGQY _{162–186}	2918.31					
4D (19.0 kDa)	EVQDSPLDACR _{39–49}	1288.57	38	19 kDa globulin precursor [<i>Oryza sativa</i> (japonica cultivar-group)]	gi 20159	21492	552
	EVQDSPLDACRQVLDR _{39–54}	1899.91					
	ERFQPMFR _{60–67}	1125.53					
	FQPMFR _{62–67}	840.39					
	RPGALGLR _{68–75}	838.51					
	MQCCQLQDVSR _{76–87}	1551.65					
	MQCCQLQDVSR ECR _{76–90}	1996.83					
	QYAAQLPSMCRVEPQQCSIFAAGQY _{162–186}	2918.31					
	QCQREVQDSPLDACR _{35–49}	1860.82					
	EVQDSPLDACR _{39–49}	1288.57					
4E (16.3 kDa)	EVQDSPLDACRQVLDR _{39–54}	1899.91	39	19 kDa globulin precursor [<i>Oryza sativa</i> (japonica cultivar-group)]	gi 20159	21492	465
	ERFQPMFR _{60–67}	1125.53					
	FQPMFR _{62–67}	840.39					
	RPGALGLR _{68–75}	838.51					
	MQCCQLQDVSR _{76–87}	1551.65					
	MQCCQLQDVSR ECR _{76–90}	1996.83					
	QYAAQLPSMCRVEPQQCSIFAAGQY _{162–186}	2918.31					
	QCQREVQDSPLDACR _{35–49}	1860.82					
	EVQDSPLDACR _{39–49}	1288.57					
	EVQDSPLDACRQVLDR _{39–54}	1899.91					
4F (8.0 kDa)	VSYAVEVGR _{112–120}	978.47	1	Os11g0546500 [<i>Oryza sativa</i>]	gi 115485845	53213	55

^a Amino acids are presented in single letter code within peptide sequences.^b Fraction C-Gt: chickpea glutelin fraction.^c Fraction R-Gt: rice glutelin fraction.

ALPVDVIANAYR, homologous to rice glutelin C precursor; this particular peptide was also identified in oat albumin, globulin and glutelin fractions (O-Ab, O-Gb and O-Gt) as a part of β -subunit from oat 12S globulin (Table 5). From such evidence, there may be some sort of genetic relationship between chickpea glutelin, rice glutelin, and oat albumin, globulin and glutelin proteins. Protein bands 4A (48.6 kDa) and 4B (28.6 kDa) from R-Gt fraction showed tryptic peptides with origin from rice glutelin precursor (15% sequence coverage) and from rice glutelin (33% sequence coverage), respectively (Kusaba et al., 2003); also the protein bands 4C (21.5 kDa), 4D (19.0 kDa) and 4E (16.3 kDa) contained tryptic peptides which showed about 37% sequence coverage corresponding to 19 kDa rice globulin precursor

(Shorrosh et al., 1992); these results suggest that SDS-PAGE protein bands of R-Gt fraction are related to rice glutelin and globulin proteins.

3.4. Identified tryptic peptide sequences with origin from oat 12S globulin

Table 5 summarizes the results of identified tryptic peptides of oat protein bands (Fig. 4) from O-Ab (lane 5), O-Gb (lane 6) and O-Gt (lane 7) fractions with origin from oat 12S seed storage globulin 1 (NCBI accession number: gi|134918; theoretical mass 58,508 Da); oat 12S globulin 1 protein is composed of 518 amino acids and belongs to 11S seed storage protein (globulin) family (Shotwell et al., 1988). The AAs

Table 5

Identified tryptic peptides of SDS-PAGE protein bands (Fig. 4) from O-Ab (lane 5), O-Gb (lane 6), and O-Gt (lane 7) fractions with origin from oat 12S seed storage globulin 1 (NCBI accession number: gil134918; theoretical mass 58,508 Da).

Peptide sequences ^a	MW (Da)	Identified in SDS-PAGE protein bands (Fig. 4)
<i>α</i>-subunit^b		
LQAFELPLR _{50–57}	972.53	6B (60.9 kDa), 6D (39.8 kDa), 6E (36.8 kDa), 6F (33.2 kDa), 6K (15.1 kDa), 7A (69.3 kDa), 7B (62.5 kDa), 7C (46.2 kDa), 7D (44.8 kDa), 7F (36.7 kDa), 7G (33.5 kDa), 7H (30.7 kDa), 7E (41.5 kDa)
LQAFELPLRQVR _{50–60}	1355.76	6E (36.8 kDa)
SQAGITEYFDEQNEQFR _{61–77}	2060.90	6D (39.8 kDa), 7A (69.3 kDa), 7B (62.5 kDa), 7F (36.7 kDa), 7G (33.5 kDa)
RVIEPQGLLLPQYHNA	2943.64	6D (39.8 kDa), 6F (33.2 kDa), 7F (36.7 kDa)
PGLVYILQGR _{86–111}	2787.54	6D (39.8 kDa), 6E (36.8 kDa), 6F (33.2 kDa), 6K (15.1 kDa), 7F (36.7 kDa), 7G (33.5 kDa), 7E (41.5 kDa)
VIEPQGLLLPQYHNAPG		
LVYILQGR _{87–111}		
SQNLKDEHQR _{144–153}	1253.61	6B (60.9 kDa), 6D (39.8 kDa), 6E (36.8 kDa), 6F (33.2 kDa), 7F (36.7 kDa), 7E (41.5 kDa)
EQQFGQNIQSGFSVQL	3252.64	6D (39.8 kDa), 6E (36.8 kDa), 7E (41.5 kDa)
LSEALGISQQAQK _{212–241}		
IQSQNDQRGEIIR _{242–254}	1555.80	6D (39.8 kDa), 6E (36.8 kDa), 6F (33.2 kDa), 7A (69.3 kDa), 7B (62.5 kDa), 7D (44.8 kDa), 7F (36.7 kDa), 7H (30.7 kDa)
<i>β</i>-subunit^c		
QNIENPK _{330–336}	841.42	5L (25.8 kDa), 6B (60.9 kDa), 6G (27.8 kDa), 7A (69.3 kDa), 7B (62.5 kDa), 7I (26.2 kDa), 7J (23.5 kDa), 7E (41.5 kDa)
RADTYNPR _{337–344}	991.48	5L (25.8 kDa), 6B (60.9 kDa), 6G (27.8 kDa), 6H (24.1 kDa), 6I (22.0 kDa), 6K (15.1 kDa), 7A (69.3 kDa), 7B (62.5 kDa), 7C (46.2 kDa), 7D (44.8 kDa), 7F (36.7 kDa), 7H (30.7 kDa), 7I (26.2 kDa), 7J (23.5 kDa), 7E (41.5 kDa)
ADTYNPR _{338–344}	835.38	5L (25.8 kDa), 7D (44.8 kDa)
VQVNNHGGTVFNDILR _{398–414}	1952.02	6G (27.8 kDa), 6H (24.1 kDa), 7D (44.8 kDa), 7I (26.2 kDa)
ALPVDVLNAYR _{461–472}	1300.71	5L (25.8 kDa), 6B (60.9 kDa), 6G (27.8 kDa), 6H (24.1 kDa), 6I (22.0 kDa), 6K (15.1 kDa), 7A (69.3 kDa), 7B (62.5 kDa), 7C (46.2 kDa), 7D (44.8 kDa), 7F (36.7 kDa), 7H (30.7 kDa), 7I (26.2 kDa), 7J (23.5 kDa), 7E (41.5 kDa)
NNRGEFGAFTPK _{483–495}	1465.69	6G (27.8 kDa), 6H (24.1 kDa), 7D (44.8 kDa), 7I (26.2 kDa)
GEEFGAFTPK _{486–495}	1081.50	6G (27.8 kDa), 7D (44.8 kDa), 7I (26.2 kDa)

^a Amino acids are presented in single letter code within peptide sequences.

^b *α*-subunit: AAs position located between 25 and 317.

^c *β*-subunit: AAs position located between 318 and 518.

sequence of this protein consists of a signal peptide at the AA position 1–24, an acidic polypeptide chain (*α*-subunit) at AA position 25–317, and a basic polypeptide chain (*β*-subunit) with AA position 318–518; a disulfide bond is observed at AA position 45–78 and an interchain (between *α*- and *β*-subunit) is observed at AA position 121–314 (Shotwell et al., 1988). Most of the protein bands O-Gb and O-Gt fractions showed tryptic peptides with origin from oat 12S seed storage globulin 1 (Table 5); as for O-Ab fraction, there was only one protein band 5L (25.8 kDa) with identified tryptic peptides (QNIENPK,

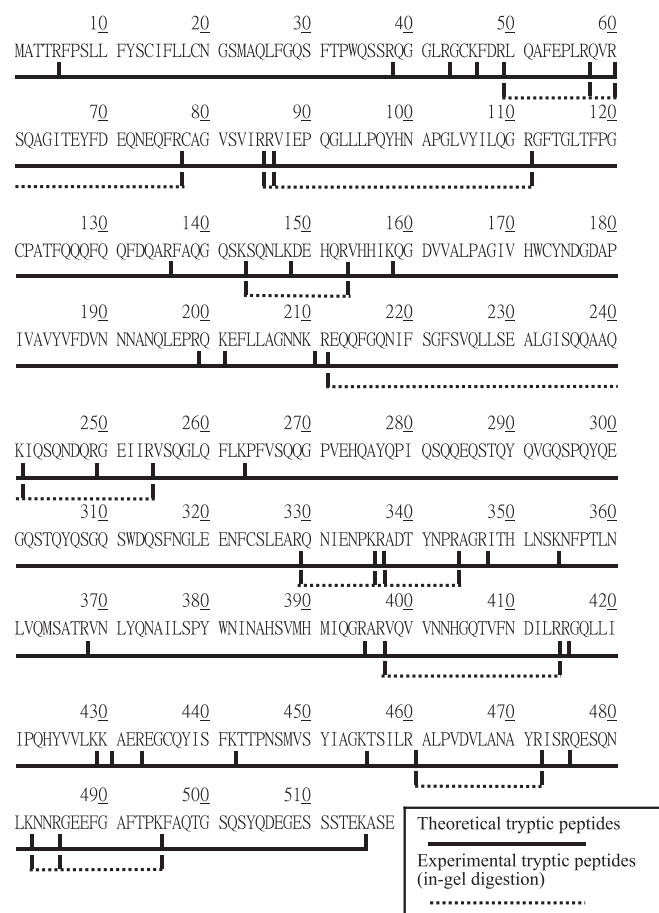


Fig. 9. Amino acids sequence of identified tryptic peptides with origin from oat 12S seed storage globulin 1 (NCBI accession number: gil134918; theoretical mass 58,508 Da).

RADTYNPR and ALPVDVLNAYR) corresponding to this protein; 8 tryptic peptides from O-Gb and O-Gt fractions are related to *α*-subunits of oat 12S globulin 1 while 7 tryptic peptides from O-Ab, O-Gb and O-Gt fractions are related to *β*-subunits of this protein.

Fig. 9 shows the AAs sequence of oat 12S seed storage globulin1 with the tryptic peptides identified from oat protein fractions during the proteomic analysis. In theory, 47 cleavage sites at either arginine (R) or lysine (K) of oat 12S globulin 1 AAs sequence are available for tryptic hydrolysis; one cleavage site at AAs position 262–263 (KP) is not available due to trypsin does not react to proline at C-terminal; 23 cleavage sites appeared in identified tryptic peptides derived from in-gel digestion. Based on the AAs sequence, 161 AAs from identified tryptic peptides out of 518 AAs is accounted for 21% sequence coverage.

4. Conclusions

SDS-PAGE results gave well defined protein bands for both chickpea and oat protein fractions (C-Ab, C-Gb, C-Gt and O-Ab, O-Gb, O-Gt, respectively); both globulin fractions (C-Gb and O-Gb) showed molecular similarities especially the observed *α*- and *β*-subunits in SDS-PAGE results. The proteomic techniques employing 1D SDS-PAGE, in-gel tryptic digestion, LC–ESI–MS/MS analysis, and online Mascot MS/MS ion search resulted in the identification of tryptic peptides sequences from chickpea, oat and rice protein fractions; chickpea and oat protein fractions (C-Gb, C-Gt and O-Gb, O-Gt) showed most tryptic peptides corresponding to chickpea legumin and oat 12S globulin 1 proteins, respectively. These identified tryptic peptides from chickpea and oat proteins can be investigated further for their potential biological properties.

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