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DYNAMIC PEPTIDE PROFILES OF GERMINATING MUNGBEAN: IN RELATION TO THEIR NATURE AND SEPARATION PATTERN

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SUMMARY

Low molecular weight peptides (3 KDa to 0.5 KDa) from different germinating phases of mung bean [*Vigna radiata* (L) Wilczek. cv. Sonali B1] were purified through ion exchange, ultrafiltration and sephadex gel permeation chromatography. The peptides were subjected to paper chromatographic and capillary electrophoretic separation, for the determination of dynamic and temporal manifestation of their occurrence. Analysis of peptides revealed mainly two groups, one of which is constitutive throughout while the other mainly appeared after 24 hrs and disappeared at 5 days of post germination phases. Amino acid analysis of these peptides indicated adequate similarities with legume storage protein. Lack of cysteine and methionine in peptide pool indicated that they are active in all different shapes suitable for transport and absorption.

***Key words*: Amino acid analysis, germinating seeds, mungbean, peptides.**

INTRODUCTION

In recent years, a vast array of bioactive peptides are isolated from different spectrum of life form and only some of these low molecular weight peptides have been characterized in detail. Peptides in plant system possess definite role in amplifying signals (Lindsey *et al*. 2002), nitrogen fixation (Mylona *et al*. 1995), cell proliferation (Matsubayashi and Sakagami 1996), generation of polarity (Souter and Lindsey 2000), differentiation, self incompatibility and mediating biotic and abiotic stress elicitation with metabolic intermediates. Concept of bioactive peptides like Systemin, Phytosulphokine, ENOD 40, CLAVATA 3 and S-Locus Factors (Ryan *et al*. 2002) trigger challenges over the classical definition of narrow viewpoint of plant hormones. The genesis of numerous small peptides in different phases of plant system is not at all random but oriented through specific molecular programs that may not be associated with central dogma of protein synthesis (Lee *et al*. 1996, Fletcher *et al.* 1999).

Now, the basic aim of this work is to explore the separation profile of peptides isolated from germinating mungbean [*Vigna radiata* (L) Wilczek. cv. Sonali B1], which is one of the important cultivated variety of mung bean with excellent aroma, taste and flavour. Till now there are very few reports in plants solely related to peptide profile or fingerprint. This may be due to technical difficulty related to isolation, purification and characterization of peptides. More often peptides are characterized through Capillary zone electrophoresis (Heintz *et al*. 2004, Wetterhall 2004), Mass-spectrometry

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(Pu *et al*. 1996), Liquid chromatography (Drykova *et al*. 2003), Paper chromatography (Rydon and Smith 1952, Suzuki *et al*. 1998), Paper electrophoresis (Bailey and Ramsaw 1973), Capillary electro-chromatography (Kasicka 2003) etc. based on the principles of the procedure and objectives of the work. This report is an analysis of impending changing pattern of peptide profile present in different germinating hrs of mungbean based on paper chromatography, capillary electrophoresis along with detection of amino acids present in these isolated peptides.

MATERIALS AND METHODS

Seeds of mungbean [*Vigna radiata* (L) Wilczek. cv sonali B1], collected from Central Pulses Research Institute (C.P.R.I.), Berhampur, West Bengal, India; were weighed (each set of 250 g) and cultured in sterile petriplates with absorbent cotton supplied with modified Hoagland solution with one-half strength of major nutrients and full-strength micronutrients. The nutrient solution was aerated continuously and changed weekly. Experiments were conducted inside a controlled environmental growth chamber with 14 hrs light period (350 µmol m^{-2} s^{-1}), 25°/20°C day/night temperature, and 80% relative humidity. The seedlings were grown for different germination time like 0h, 8h, 24h, 32h, 40h, 48h, 56h, 64h, 72h, 5days, 6days and 7days for specific experimental objectives.

Extraction: Mungbean seedlings (100 g) of each set were washed thoroughly with 0.2% sodium hypochlorite solution followed by washing with distilled water to avoid contamination. The pieces of seedling were cryo-crushed and extracted with measured amount of chilled distilled water at 4°C. The extracts were centrifuged at 10,000 rpm for 30 min with protease inhibitor PMSF at 4°C. The supernatant was collected and stored in deep freeze (-40 °C) for further study.

Ion exchange chromatography: The extracts were purified through cation (Tsunasawa and Narita 1982) and anion exchange resin (Watanabe *et al*. 2003) (Dowex 50 and Dowex 1, Sigma Chemical Co., USA), filled in two-glass column (60 cm x 2.9 cm, 1.6 meq/ml). Freshly prepared 3N ammonia and 1N HCl were used for elution of peptides from the cation and anion exchanger column, respectively. The ammonia and HCl were made free from extract solution through a liquid nitrogen trap fitted to a lyophilizer (Lyolab BII). The whole extract was freeze dried to smaller volumes. Further purification was performed through acidic (pH-5.50) aqueous ether fractionation for removal of hydrophobic components and phenolics from the extracts. After phase separation aqueous layer were taken for ultra filtration.

Ultra filtration: The lyophilized material obtained from each set of respective germination hrs was separately put through ultra-filtration through via millipore stirred cell fitted with 10,000 Da (YM10, Amicon), 3000 Da (YM3, Amicon) and 500 Da (YC05, Amicon) cut off filter paper separately and the filtrate between 3000 to 500 Da were collected. Precautions were taken for removal of amino acids from plant extract by using 500 Da cut off ultrafiltration for three times in each case. The ultrafilterd samples were lyophilized and dissolved in 10 ml distilled water (for each set) and stored in deep freeze (at - 40 °C)(Miklashevichs *et al*.1996).

Paper chromatography: Each isolated peptide solution of 100 µl (1 g fresh weight equivalent) was loaded on to Whatman No-1 chromatography paper (size-46 cm x 57 cm, thickness-0.16 mm), and separated by descending chromatography with two solvents separately [solvent1; isopropanol : ammonia : water :: 10 : 1 : 1 (v/v) and solvent 2; n-butanol : acetic acid : water :: 4 : 1 : 1 (v/v)]. The papers were stained with freshly prepared ninhydrin location reagent (Friedman 2004). The retardation factor (R_f) values were determined.

Purification of semi purified extracts through sephadex LH-20 column: Semi purified extracts (2 ml) were loaded on Sephadex LH-20 column (80 cm x 3 cm; volume-566 ml), fitted with ISCO fraction collector, peristaltic pump and UV-recorder (Andrews 1965). Samples were eluted with 30% ethanol at a collection speed of 190 drops/tube (approximately 5ml/tube), drawn by pump set at a speed of 60 digit (ISCO WIZ pump) 30 ml/h. Recorder was set at 3 cm/h; 0.1 O.D full scale, with UV monitoring range at 280 nm. The semi-purified fractions were collected in 200 tubes. After removal of void volume (first 24 tubes), tubes were grouped into four major fractions (25 to 50 tubes for Fraction-1, 51 to 100 tubes for Fraction-2, 101 to 150 tubes for

Fraction-3 and 151 to 200 tubes for Fraction-4) and lyophilized separately. Finally the lyophilized peptides were mixed with suitable volume of distilled water and used in chromatography or electrophoretic techniques for the purpose of detection. After LH-20 fractionation of peptide mixture of different germinating hrs, four fractions of peptides of 32 h and 6 days were again subjected to one dimension paper chromatography with two different solvents (solvent-1 and 2) separately.

Two-dimension paper chromatography was performed with LH-20 purified peptides. At first, the last three fractions of LH-20 were mixed together in equal volume, then 100µl of that was spotted on to Whatman No-1 chromatography paper and separated by descending chromatography with two different solvent mixtures respectively in two arms [solvent-1 in short arm and solvent-2 in long arm]. Then papers were stained with freshly prepared ninhydrin location reagent (Friedman 2004).

Capillary zone electrophoresis: After purifying through LH-20 gel exclusion chromatography, the peptides were subjected to capillary electrophoresis, Beckman P/ACE system 5010. 60µl of sample was loaded in each case (with 50 sec injection time), detection wavelength 214 nm, used neutral gas nitrogen, capillary volume 50µm x 47cm (neutrally coated), voltage-18KV (8.4 µamps, temperature-20°C) detection time of 5 seconds was monitored. eCAP™ citrate buffer pH-3 (20µM citrate) was used as running buffer assuming the isoelectric points of peptides are greater than pH-4 using normal polarity. eCAP™ Orange G (0.1% aqueous solution) is used as reference marker. Peak height (Cx=k.Hx), peak area (Cx= k.Ax) and area percentage were calibrated from electrophoregram using software System Gold Version 810. Molecular weight determination was performed by using standard peptides: Insulin (MW-5777.6), Insulin chain-A (MW-2531.6), Insulin chain-B (MW-3495.9), Somatostatin (mol.wt. -1637.9) [Sigma Chemical Co. USA] and CNBr treated lysozyme fraction A (MW-1025.61) with the help of Smith's Statistical Package (Version-2.5 of Gary Smith) by $\log_{10}$ MW vs. 1/RMT graph. Chromatograms of different peptides of germinating mung bean seedlings were presented graphically using Microsoft Excel.

Amino acid analysis: After total chemical hydrolysis of peptides isolated from 6 day's mung bean seedlings, amino acid analysis was done according to phenyl isothiocyanate – derivatised method with the help of Applied Biosystem (Model 476A) amino acid sequencer of protein (C_{18} column with reverse phase support, 220 mm X 2.1 mm I.D., pH stability 3-12, run time 20 min, solvent – acetonitrile : water :: 60 : 40, flow rate 1ml/ min., wave length - 250 nm).

RESULTS

The overall expression of one dimension paper chromatography is thoroughly documented in Fig. 1A. From that appearance, it is quite prominent, that in case of solvent-1, the separation pattern is little bit better than that of solvent-2, which indicates significantly towards the nature of peptides (better resolved in two dimensional paper chromatography). Though the same amount of 1 g fresh weight equivalent of purified plant extracts were spotted on chromatographic paper, it was observed that the amount of peptides present in 24h, 32h, 40h, 48h, 56h, 64h and 72h was drastically high than that of 8h, 5 days and 6 days (supported by apparent visibility and intensity of ninhydrin stained spots). Now, in both the solvents, some consistent characters were observed throughout all the germination hours – like, a definite gap zone (from, R_f - 0.25 to 0.35, in solvent-1 and R_f -0.4 to 0.5, in solvent-2); good number of peptides bear proline or hydroxy-proline in their amino terminal as seen by yellow colour of spot; in solvent-1, a very odd spot was present with highest R_f value (0.739) in case of 64h; in solvent-2, peptides of 8 h had a very weak separation. The complete result is mainly documented in Fig. 1B. The overall expression shows that, the major amount of distinguishable peptides came within fraction-1 and 2; though in fraction 1, some spots overlapped.

Two-dimensional paper chromatography was done with peptide mixtures of last three LH -20 fractions (just to make the peptide fingerprinting more dependable) of different germination hrs with solvent-1 and solvent-2, applied in two different arms. The peptides of 0, 8, 32, 72 hrs and 6 days separated in to 10, 11, 19, 16 and 13 spots, respectively (Fig. 1C) whereas the peptides of 24, 40, 48, 56, 64, 72 hrs and 5 days separated in to 14, 16,

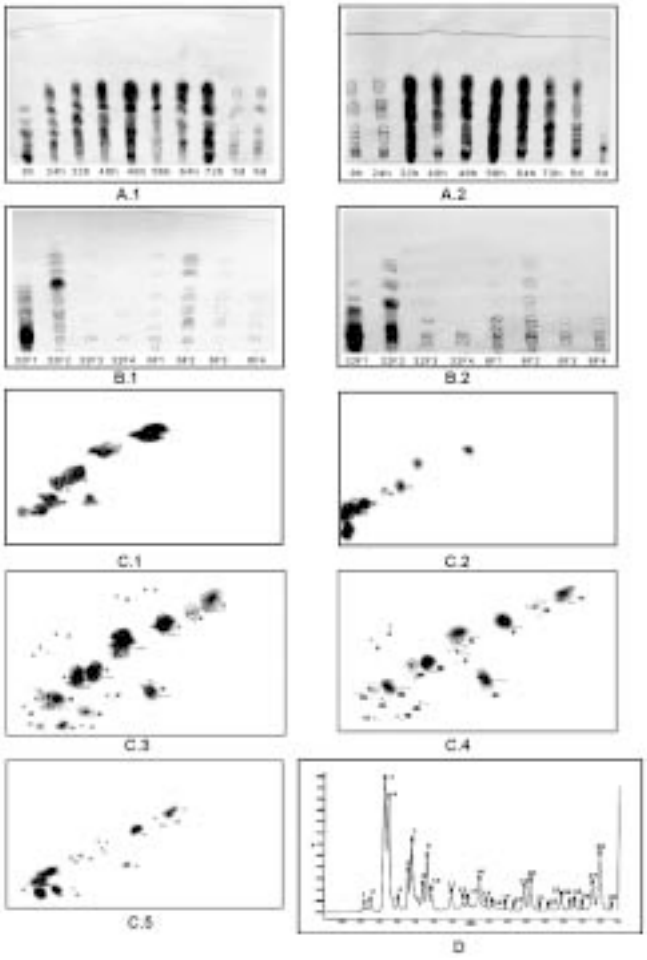

Fig. 1A-Comparative one dimension paper chromatography of isolated peptides from different hours of germination: A1–in isopropanol : ammonia : water :: 9 :1 : 1 (v / v / v), A2–in n-butanol : acetic acid : water :: 4 :1 : 1 (v / v / v); B– Comparative one dimension paper chromatography of different LH–20 separated fractions of isolated peptides from 32 h and 6 d of germination: B1–in isopropanol : ammonia : water :: 9 :1 : 1 (v / v / v), B2–in n-butanol : acetic acid : water :: 4 :1 : 1 (v / v / v); C–Two dimension paper chromatography: C1–peptides of 0 h, C2–peptides of 8 h, C3-peptides of 32 h, C4–peptides of 72 h, C5–peptides of 6 d; D–Amino acid analysis of isolated peptides of 6 d through RP–HPLC based amino acid analyzer; Peak number 1 -, 2 -, 3 aspartic acid, 4 glutamic acid, 5 -, 6 serine 7 glycine &/or histidine, 8 threonine, 9 alanine & / or proline, 10 alanine & / or proline, 11 ammonia, 12 tyrosine, 13 -, 14 valine, 15 -, 16 methionine, 17 cysteine, 18 -, 19 isoleucine, 20 leucine, 21 -, 22 -, 23 phenylalanine, 24 -, 25-, 26 -, 27 lysine, 28 lysine, 29 -

22, 14, 17, 16 and 13 spots, respectively (Fig. not included).

Major peaks were detected mainly in fraction-2 and fraction -3 by Capillary electrophoresis. Peptides of fraction – 1, generally execute some overlapping peaks with trailing effect and fraction – 4, contained lowest number of peaks. (Some are documented in Fig. 2A to C; and complete electrophoregram, prepared on the basis of total capillary electrophoretic responses, in Fig. 2D).

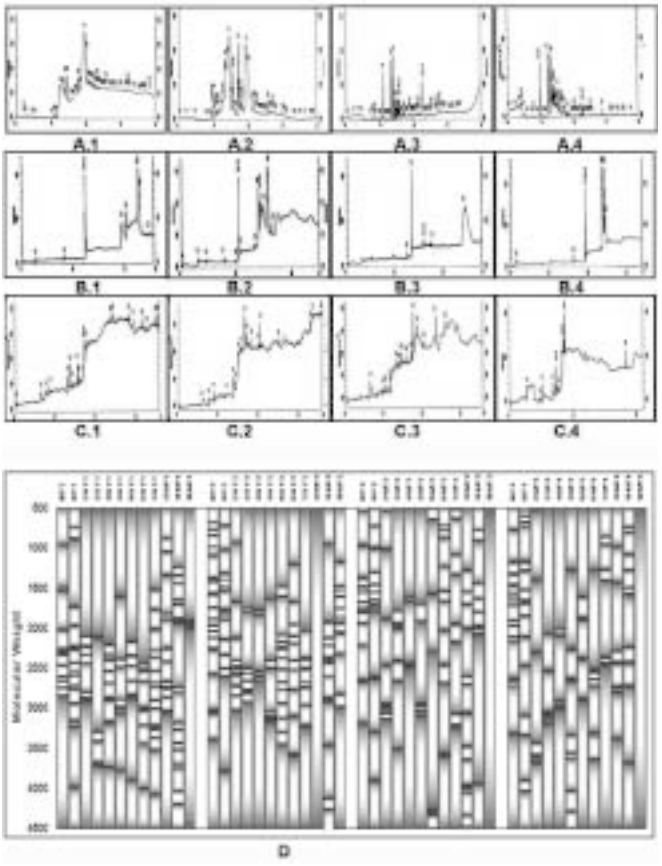

Fig. 2. (A-C)-Capillary electrophoretic representation of different LH-20 fractions of isolated peptides of some germination hours-A1-peptides of fraction-1 of 0 hours, A2-peptides of fraction-2 of 0 hours, A3-peptides of fraction-3 of 0 hours, A4-peptides of fraction-4 of 0 hours; B1-peptides of fraction-1 of 32 hours, B2-peptides of fraction-2 of 32 hours, B3-peptides of fraction-3 of 32 hours, B4-peptides of fraction-4 of 32 hours; C1-peptides of fraction-1 of 6 days, C2-peptides of fraction-2 of 6 days, C3-peptides of fraction-3 of 6 days, C4-peptides of fraction-4 of 6 days; (D) electrophoregram, of all the germination hours, prepared on the basis of capillary electrophoretic representation.

Amino acid analysis of peptides from 6 day's mung bean seedlings showed interesting results. Amino acid analysis showed sixteen amino acids in isolated peptides. The amount of aspartate-asparagine pool and glutamate-glutamine pool were observed to be highest. The amino acids like serine and threonine, which contain aliphatic hydroxyl group, expressed moderately. Tyrosine and phenylalanine were present in low amount. Cysteine and methionine were almost insignificant. (Fig. 1D)

DISCUSSION

Protein fingerprinting, in plants, has wide application in different fields of study. This can be treated – to distinguish different legume cultivars or species (Ahmad and Slinkard 1992, Gardiner and Forde 1992, Gepts *et al*.1992, Takehisa *et al*. 2001) as biological marker in biodiversity study (Tomooka *et al*. 1992) or in functional aspect of spatial and temporal developmental context (Das *et al*. 2006).

Peptides are also one of the most potent groups of bioactive molecules in the plant system (Lindsey *et al*. 2002, Mandal *et al*. 2006). They perform different biological activities with disciplined genesis (Lee *et al*. 1996, Fletcher *et al*. 1999). So, peptide fingerprinting is also a controlled and organized representation of its own kind with definite execution of internal metabolism.

In the present study, the primary focus was on the temporal difference in peptide pattern of germinating mung bean based on paper chromatography and capillary zone electrophoresis. The comparative analysis of peptide profile reveals mainly two groups of peptides. One of them is evident throughout the germination period. They might be called as "housekeeping peptides" with lower range of partition coefficient, as they showed a better separation in alkaline solvent [pH 12.11] (Lala 1981) in all chromatograms. Actually they are more prominent in paper chromatograms with LH-20 separated fractions of 32 h & 6 days and also in electrophoregram (32 h and 6 days of germination periods are chosen because of their diverse nature; in 32h, the developing embryo is dependent on storage food reserve but in 6 days, it becomes nearly independent). These housekeeping peptides mainly ranges from 1500 Da to 3500 Da (consistency of peaks is evident throughout the germination in Capillary Zone Electrophoresis). From one dimension paper chromatography, it was evident that a perceptible amount of these peptides contains proline or hydroxyproline in their amino terminal (Conn *et al.* 1999). House keeping proteins with a wide range of molecular weight have been already reported in mung bean (Das *et al*. 2006).

The other groups of peptides appeared from day one of germination and again nearly disappeared at later stages. This shows that, they are mainly synthesized during the peak period of germination. These peptides are amphoteric in wide range of pH (better resolved in two dimension paper chromatography) with moderate range of partition coefficient. The major part of these peptides came within first 150 tubes of sephadex Gel Exclusion Chromatography and their molecular weight ranges from 500 Da to 3500 Da. Depending on the nature and molecular weight, it may be predicted that these types of peptides are in most active form for transport, absorption and apoplastic migration. During germination, enzymatic hydrolysis of storage proteins forms a reservoir of small peptides which are translocated to the growing embryo for nutritional supply. Similar phenomenon is observed in pea seed germination (Liu *et al*. 1996). Transport of these kinds of nutritional peptides has already been demonstrated in some monocots and *Arabidopsis* with specific peptide transporter (Waterworth *et al*. 2000, Stacey *et al*. 2002). Though, in Legumes, the picture is not so clear but similarities can be expected. Even, a large number of peptides have been recognized for their developmental phase related signaling behaviour (Ryan *et al*. 2002). So, a number of those specific types of peptides affecting germination might be present in this group.

Amino Acid analysis of 6 day's germinating mung bean support the above view. Seed proteins contain high level of amides (glutamine and asparagine) and are deficient in methionine and cysteine (Shotwell and Larkins 1989). Similarities in this nature for peptides might not be an unusual phenomenon. These peptides may be present in all different forms due to lack of disulphide bonds as established from amino acid analysis where cysteine was present in insignificant amount.

In conclusion, the present approach allowed us to recognize the abundance and dynamic shifting of low molecular weight peptides during various phases of seed germination and seedling establishment in *Vigna radiata*. Studying changes of individual peptide spots over the seedling growth period enable classification according to their existence and separation pattern. First group of peptides present throughout the germination periods were basically housekeeping in nature. Second group which increased gradually up to the stage of development of photosynthetic machinery and independence, included peptides may perhaps be involved in nutritional supply. Another group where spots appeared transiently just at the time of conversion from heterotrophic to autotrophic nature, included peptides possibly involved in developmental phase specific bioactivity.

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