Journal of Phytology 2011, 3(7): 57-63
ISSN: 2075-6240
www.scholarjournals.org

JOURNAL OF PHYTOLOGY

www.journal-phytology.com

Towards the Mungbean 'Peptidome': Assessing the Bioactivity of Low Molecular Weight Peptides Isolated from Germinating Seeds

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Article Info

Article History

Received : 16-02-2011
Revised : 14-03-2011
Accepted : 15-04-2011

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Summary

Numerous studies have established that peptides function as signaling molecules, and play significant role in cell-to-cell communication in almost all living organisms. In plants, the impact and importance of 'peptide signaling' in regulation of growth and development has triggered challenges over the classical definition and function of 'traditional plant hormones'. In the present study, we isolated the complete '*peptidome*', consisting of low molecular weight peptides in the range of 3 kDa to 0.5 kDa, of germinating mungbean [*Vigna radiata* (L) Wilckzek. cultivar Sonali B1] at different hours of germination. A comparative profiling of the peptidome was done through thin layer chromatography, followed by an evaluation of their bioactivity *in vitro*. Majority of peptides were found to be abundantly present at 24, 48 and 72 h of germination; however, showed a significant decrease at 120 and 144 h of germination. Bioactivity analysis revealed that peptides isolated at 24, 48 and 72 h, but not 120 and 144 h of germination showed positive and inductive control over α-amylase activity and cell division *in vitro*. Our results suggest towards the conditional synthesis of specific types of low molecular weight peptides during active germination in mungbean plants.

Introduction

In 1902, Emil Fischer, for the first time, introduced the term 'peptides' (possibly derived from pepsis = digestion or peptones = digestion products of proteins) for those small sized bio-molecules that resemble 'proteins', except that the latter one is of higher molecular weight. In general, bio-molecules of 50 or more amino acids are generally considered as proteins, and those containing less number of amino acid residues are designated as peptides. Although, studying proteins has developed into the field of proteomics [1,2]; understanding peptides, both chemically and biologically, has only recently become a major point of interest for researchers worldwide. Even so, a new branch of science, dealing with the total peptide content (i.e. '*peptidome*') of a cell, tissue or organism, namely '*peptidomics*' has emerged in recent years [3,4].

Plants possess a vast array of bioactive peptides that play very important roles in various aspects of plant growth and development [5]. A significant number of these peptides are secretory in nature, and act as local signals which promote cell-to-cell communications [6]. Yet, our knowledge on plant peptides is quite recent. Systemin, an 18 amino acids peptide, was the first plant peptide to be discovered from the wounded leaves of tomato plants by Gregory Pearce and colleagues in 1991. Systemin is an intracellular signaling peptide, synthesized from its 200-amino acid precursor- prosystemin, and acts as defense signal in Solanaceae species [7]. In the following years, discovery of some other important plant peptides such as Phytosulphokine (PSK) that induces cellular dedifferentiation and proliferation [8], SCR/SP11 (S-locus cysteine rich protein/ S-locus protein 11) that determines the self incompatibility of pollen [9], and Clavata (CLV) that regulates the shoot apical meristem (SAM) growth [10] have increased our understanding of the plant's '*peptidome*' [11].

However, to date, majority of the research has been focused on single and/or specific peptides. Researchers have either isolated the peptides or synthesized them chemically, with an aim to study their bioactivity in plants [5, 7, 11, 12]. On the other hand, only a few studies have been reported dealing with the '*peptidome*' content of the plant. For example, Gara et al. [13] extracted total peptides from oat plant (*Avena sativa* L.) and analyzed them by mass spectrometry; their results predicted that most of the low molecular weight peptides could be fragmented parts of enzymes and other functionally important plant proteins [13]. It should be noted that the authors could not fully identify or sequence these peptides due to lack of the oat genome sequence. In another study, Ivanov et al. [14] isolated and identified total peptides from early developmental stage (filamentous or protonemal) of a moss, *Physcomitrella patens* (Hedw.) B.S.G., and showed that these peptides were mostly derived from proteins localized in plastids [14]. Mandal et al. [15] also extracted total peptides from mungbean seedlings, and studied their separation profiles

using techniques of paper chromatography and capillary electrophoresis [15]. In spite of these studies, there was almost no information on the role or possible impact of the '*peptidome*' on plant metabolism itself. During the writing of this paper, we came across the work of Ghosh et al. [16], who working with peptides derived from 7 days-old wheat plant, demonstrated the positive effect of wheat peptides on α-amylase activity in the *Hordeum vulgare* L. seeds and stomatal opening of *Cololcasia esculanta* L. *in vitro*; their results concluded that naturally occurring peptides can modulate various physiological processes in plants [16].

With this background, we examined the dynamic occurrence of low molecular weight peptides (termed '*peptidome*'), specifically ranging between 3 kDa to 0.5 kDa, in germinating mungbean seeds, and their potential role in particular physiological processes. We hypothesized that the isolated peptides could mimic the action of signaling molecules or plant hormones, and demonstrated positive control on mungbean seed germination. These results are an initial report on the dynamism of germinating seed '*peptidome*' of mungbean, and will pave the way for their further characterization by high-throughput *proteomics/peptidomics*.

Material and Methods

Plant Material and Growth Conditions

Mungbean [*Vigna radiata* (L) Wilckzek.cultivar Sonali B1] seeds were collected from the Central Pulses Research Institute (CPRI), Berhampur, West Bengal, India, and used in the present study. Sonali B1 is a highly popular cultivar grown for its high quality seeds with higher nutritional value. Equal amount of properly surface sterilized seeds (each set of 250 g; n = 6) were germinated in sterile Petri dishes with modified Hoagland solution of one-half strength major nutrients and full strength micronutrients (containing – 1M KNO_3, 1M Ca $(NO_3)_2$, Iron, 1M $MgSO_4$, 0.5M NH_4NO_3, and full strength of H_3BO_3, $MnCl_2$, $ZnSO_4$, $CuSO_4$, Na_2MoO_4, KH_2PO_4) for 24, 48, 72, 120 and 144 h. The nutrient solution was replaced at definite intervals, with fresh nutrient solution of same strength. The whole experiment was performed inside a controlled environmental growth chamber with 14 h light period (350 µmol m^{-2} s^{-1}), 25°/20°C day/night temperature, and 80% relative humidity. All the experimental sets were maintained contamination free, up to the completion of the experiment.

Isolation of Low Molecular Weight Peptides

Extraction of Plant Material

Different sets of mungbean seeds, maintained up to 24, 48, 72, 120 and 144 hof germination, were taken for peptide isolation. Germinated seeds (100 g of each set) were washed thoroughly and carefully with mild strength detergent solution for 20 min followed by 0.2% sodium hypochlorite solution for 10 min, and finally with sterile distilled water for 30 min. These steps were followed to avoid any type of contamination to the sample. The germinated seeds were cryo-crushed with liquid nitrogen (liq. N_2) and extracted with 3 volumes of pre-chilled sterile distilled water at 4°C. The extracts were centrifuged at 10,000 rpm for 30 min with protease inhibitor PMSF (phenyl methyl sulfonyl fluoride) at 4°C. PMSF was added to the extract just before centrifugation with a concentration of 0.8 mM. The supernatant or crude extract was collected and stored in aliquots at -80°C for further purification and/or analysis.

Ion Exchange Chromatography

The primary purification of crude extract was performed using ion exchange chromatography in two steps: i) The extract was passed through cation exchanger resin Dowex 50 (900 meq, Sigma Chemical Co., USA; filled in glass column 60 × 2.9 cm. 1.6 meq mL^{-1}). During this purification step, anionic hormones like indole acetic acid (IAA), gibberellic acid (GA_3), and abscisic acid (ABA) were removed from the column by washing sequentially with sterile distilled water, ethanol (50%) and 1-bed volume of sterile distilled water. Basic compounds like cytokinin, amino acids, peptides and related compounds were retained in the column. Resin was collected from the column in a beaker (3 L in size) and approximately 500 mL sterile distilled water was added. Cation exchange resin was neutralized with 50% liquid ammonia (1:1:: water:ammonia) with constant agitation to avoid any exothermic reaction and reloaded again into the column. This was done very carefully in a cold room at 4°C to avoid any loss of bioactivity of the target molecule. The cation exchange resin was further eluted with half-bed volume of 3 N NH_4OH in the cold [17] and washed with sterile distilled water. The whole elution was lyophilized using a liq. N_2 trap-fitted lyophilizer (Lyolab BII, Heto-Holten, Denmark). ii) The lyophilized material was dissolved in sterile distilled water with slightly acidic (pH 5 – 6) and passed through anion resin Dowex 2 (700 meq, Sigma; filled in glass column 60 × 2.9 cm. 1.6 meq mL^{-1}), washed, neutralized and eluted with 1N HCl according to the above procedure mentioned in cation exchange resin neutralization. Basic compounds and cytokinin were removed from the column. The eluent of anion resin was again lyophilized as above. At this step, the eluent passed through cation and anion resins are free from any electrolytes, and only amphoteric compounds like amino acid, peptides were present in the solution. The concentrated aqueous acidic liquid (pH 5.5) was washed four times with equal volume of peroxide free ether to remove traces of IAA, GA, ABA etc., and was discarded. The isolated heterogeneous peptide pool was further lyophilized and kept at -80°C for subsequent purification.

Ultrafiltration

The isolated, lyophilized, partially purified heterogeneous peptide pool of germinating mungbean was ultra-filtrated using a Millipore stirred cell fitted with 10,000 Da (YM10, Amicon, Millipore Corporation, USA), 3000 Da (YM3, Amicon) and 500 Da (YC05, Amicon) cut-off filter membrane separately, and the filtrate between 3000 to 500 Da was collected. Precaution was taken for removal of amino acids from the isolated heterogeneous peptide pool, by using a 500 Da cut-off ultra-filtration step thrice in each case. The ultra-filtered extract of heterogeneous peptide pool (between 3000 – 500 Da) was then lyophilized completely, and dissolved in 10 mL sterile, pre-chilled distilled water and stored at -80°C for further analysis.

Purification of Extracted Semi-purified Heterogeneous Peptide Pool (3000 - 500 Da) through SephadexLH-20 Column

Semi-purified extract of heterogeneous peptide pool (3000 – 500 Da) (2 mL) was loaded onto a Sephadex LH – 20

column (80 cm × 3 cm; volume – 566 mL), fitted with ISCO fraction collector, peristaltic pump and UV-detector [16]. Samples were eluted with 30% ethanol at a collection speed of 190 drops $tube^{-1}$ (approximately 5 mL $tube^{-1}$), drawn by pump set at a speed of 60 digit (ISCO WIZ pump) 30 mL h^{-1}. Recorder was set at 3cm h^{-1}; 0.1 O.D. full scale, with UV monitoring range at 280 nm. The elution was collected in 200 tubes. After removal of void volume (the first 24 tubes), remaining tubes were grouped, and lyophilized. Finally the lyophilized volumes were mixed with 10 mL pre-chilled, sterile distilled water, and kept at -80°C for further analysis.

Thin Layer Chromatography (TLC) of Isolated Peptides

100 µL (1 g fresh weight equivalent) of each isolated peptide solutions were slowly and carefully loaded on pre-coated TLC Silica gel 60 plates (Merck Chemicals, Germany) with a capillary tube, so that the diameter of the loaded spots did not exceed 5 mm. The loaded peptide spots were properly dried and separated by ascending chromatography with a solvent mixture - n-butanol: glacial acetic acid: water:: 4:1:1 (v/v/v) [18]. Post-chromatographic run, the TLC plates were stained with freshly prepared ninhydrin location reagent [19], and dried in a hot air oven at 60°C for 30 min. The obtained profile was then documented, and retardation factor (R_f) values were calculated.

Evaluation of Bioactivity

Amylase Induction Assay

Wheat seeds (*Triticum aestivum* L. cv Sonalika) were collected from National Seed Corporation (NSC) authorized dealer Jaishree seeds, Malda, India. Embryo-less half seeds of wheat (30 in number) were placed in sterile Petri dishes (11 cm diameter) lined with blotting paper. The half seeds were separately incubated for 48 h at 28°C, with peptide solutions obtained at different hours of germination, GA_3 of different molarities (which are– 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} M), and double distilled water as control. Each experimental set was performed with six biological replications (n = 6). After incubation, the seeds were taken out from each set, and crushed separately with 4 mL pre-chilled 100 mM Na-phosphate buffer (pH 7.0) containing 0.1 m Methylene diamine tetraacetic acid (EDTA), 1% (w/v) polyvinyl pyrrolidone (PVP) and 0.5% (v/v) Triton X-100; at 4°C. The homogenate was centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant was utilized as crude enzyme extract. One mL of the enzyme extract was then incubated with 1 mL of 1% starch solution for 3 min at 25°C. After the incubation, activity of amylase was studied as reducing sugar produced through 3, 5-dinitro salicylic acid method [20].

Effect on Cell Division

Commercially available fresh and healthy onion bulbs (*Allium cepa* L.), without any treatments, were obtained from Kolkata, India; and cultured aseptically in sterilized sand (properly saturated with modified Hogland solution) for emergence of root up to 48 h at room temperature. After that, bulbs with only healthy growing roots were selected, and incubated for 12 h at room temperature, with peptide solutions of different germination hours, and double distilled water as control. Each experimental set was performed with six biological replications (n = 6). At the end of incubation 5-6 root tips from each bulb were prepared for microscopic examination. The root tips were fixed and macerated in a solution of 45% acetic acid (9 parts) and 1M HCl (1 part) at 50°C for 5 min, followed by squashing in a 2% orcein stain in45% acetic acid. Slides were kept in a freezer and readily documented under compound microscope (Olympus BX51, Japan).

Statistical Analysis

All the results were processed as mean and standard error of mean, and then subjected to one-way ANOVA for assessing the significance of quantitative changes in assessed parameters due to the treatments with peptide solutions of different germination hours. Duncan's multiple range test was performed as post hoc on parameters subjected to ANOVA (only if the ANOVA was significant). All the statistical tests were performed using SPSS software (SPSS Inc., version 16.0).

Results and Discussion

1-D Thin Layer Chromatographic Separation Manifest a Dynamic Occurrence of a Major Group of Peptides during Early Hours of Germination in Mungbean Seed Peptidome

The invention of two-dimensional (2-D) gel electrophoresis by Patrick H O'Farrell and his contemporary workers in the 1970s, and henceforth, its wide application by researchers has notably enriched the field of '*proteomics*' [21-25]. However, the resolution power of 2-D gels is a limiting factor for the identification of smaller proteins (especially with molecular weight lower than 5-10 kDa), i.e. - peptides. Therefore, researchers have used the '*peptidome'* profiling approach, mostly through different chromatographic and mass spectrometric techniques [15; 26-29]. Due to method simplicity, easy detection, and least interfering factors; TLC (especially layers made with silica gel or cellulose) has obtained a wide application in peptide analysis. In this technique, various solvent combinations have showed suitability for peptide profiling, including those designated for amino acid separation. Nevertheless, the most universally accepted solvent combination, for peptide study, consists of n-butanol, acetic acid, and water (4:1:1) [18].

For our study, we used TLC (using pre-coated TLC silica gel-60 plates) for profiling total peptide pool (between 3 kDa to 0.5 kDa) isolated from different hours of germination of mungbean seeds; with n-butanol, acetic acid, and water (4:1:1) (see section 2.3.) as running solvent. The nature and separation of isolated peptides in 1- dimensional TLC, were almost similar for 24, 48 and 72 h of germination, but changed significantly for 120 and 144 h of germination (Fig 1). Fewer peptide(s) spots were observed at 120 and 144 h, as compared to the other three germination hours. Mandal et al. [15] also reported similar pattern in the separation of low molecular weight peptides, through one - and two – dimensional paper chromatography and capillary electrophoresis, in their study [15]. Their results revealed mainly two groups of peptides; one of which remained throughout the time points examined; while the other mainly appeared after 24 h and disappeared at 5 d or 120 h of germination. Based on their results, Mandal et al. [15] hypothesized that the second group of peptides, which appeared after 24 h of germination and disappeared at 120 h, might have some important role during the seed germination in

mungbean [15]. Our results further confirmed the data of Mandal et al. [15], and we proceeded to the next step in our study, namely, the assaying of the bioactivity of theses peptide pools to understand their dynamic appearance during the active germination of mungbean seeds, and to correlate the separation pattern with activity.

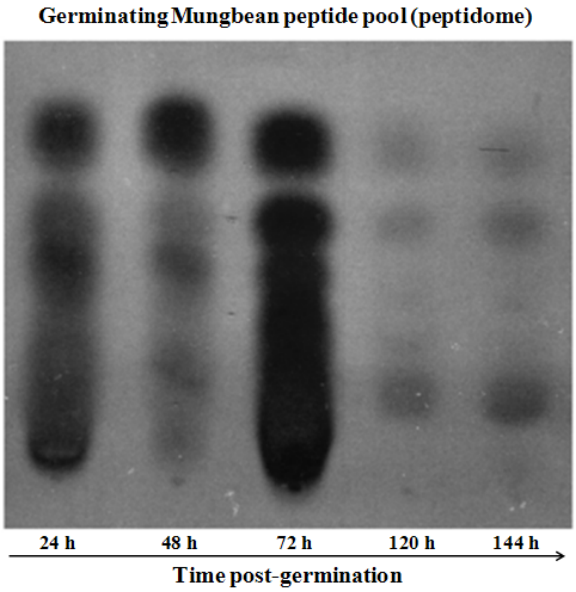

Fig1.One-dimensional thin layer chromatographic separation of isolated mungbean 'peptidome' (ranging between 3 kDa to 0.5 kDa) show dynamic occurrence of major peptides, during the germination of mungbean seeds

Bioactivity Analysis of Dynamically Occurring Peptides Indicates their Active Participation in Controlling Germination Process at In Vitro

Amylase activity (specifically, α-amylase) and cell division are two indispensable events of the seed germination process. Therefore, we confined our assays to these two events *in vitro*. As the peptides were isolated from mungbean in this study, we choose wheat seeds for examining the α-amylase activity and onion roots (root tips) for the cell division study (see section 2.4.), in order to make the results unbiased and free from any *in vivo* factors.

Low Molecular Weight Mungbean Peptides from Early Hours of Germination Appreciably Induce α-amylase Activity, and Mimic the Gibberellic Acid Action

In general, during seed germination, GA_3 acts as a signaling molecule and induces the transcript level of α-amylase gene in aleurone layer cells contributing to subsequent starch degradation in the endosperm to provide sufficient nutrition and energy to the growing embryo [30]. For the α-amylase activity study, we used embryo less half seeds of wheat in order to prevent any *in vivo* supply of GA_3 (see section 2.4.1.).Our results showed that the externally applied GA_3significantly induced α-amylase activity as compared to control, respectively (Fig 2). The concentration of 10^{-5} M of GA_3, found to be most effective among all doses tested. Interestingly, the externally applied peptides at 24, 48 and 72 h of germination, also actively induced the α-amylase activity as compared to control, respectively (Fig 2).Among all the applied concentrations, 10 mg fresh weight equivalent peptides have shown the highest stimulation on α-amylase activity. Although, the peptides at 120 and 144 h of germination did not exert significant effect on α-amylase activity as compared to other three germination hours and GA_3, respectively, their values were significantly higher than the controls (Fig 2). This result suggests that, the group of differentially expressed peptide(s), within the isolated peptide pool of first three (i.e. - 24, 48 and 72 h) and last two (i.e. - 120 and 144 h) germination hours, are appreciably mimicking the hormonal (in this case, GA_3) action in mungbean plants during germination. Even the profuse appearance of these specific groups of peptide(s) in TLC separation also supports this view (Fig 1).

Ghosh et al. [16] have reported similar induction of α-amylase activity in embryo less barley seeds treated with low molecular weight peptides, isolated from wheat seeds at 168 h (or 7 d) of germination [16]. However, our findings contrasted their results, and thereby, suggested towards a conditional regulation in synthesis of these bioactive peptide(s) in plants. It is suggested that this difference in results may be due to the use of two different plant materials, including inherent differences in the germination rate and processes, therein. Mastoparan, a cationic amphiphilic tetradecapeptide isolated from the wasp venom, and its analogs are well known to activate mammalian 'heterotrimeric G proteins' even in the absence of signal-receptor molecules, and can be taken up by intact cells [31]. Fujisawa et al. [32] have reported that mastoparan 7 (an active analog of matoparan) acts similarly in plants, and induces α-amylase activity via the heterotrimeric G protein in rice embryo-less half seeds, as seen with GA_3 [32; for details see review 33].Based on the above reports, it is tempting to speculate the occurrence of mastoparan 7-like peptide(s) in the isolated peptide pool of mungbean, in our study.

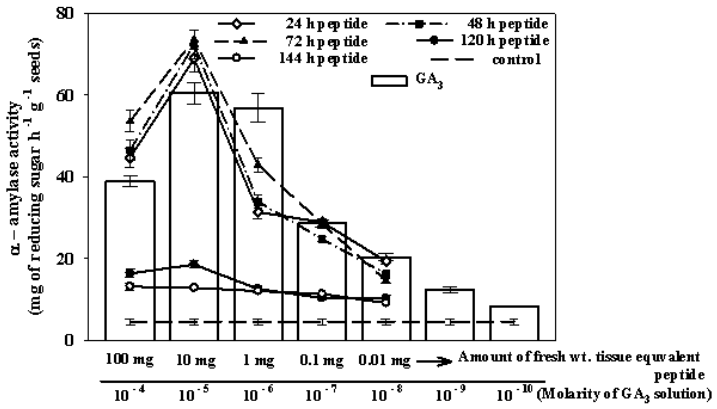

Fig2.Effect of lowmolecular weight mungbean peptides (3 kDa- 0.5 kDa) andgibberellic acid (GA_3)on the α-amylase activity of embryo less half seeds of wheat,*in vitro*.Values represent mean ± SE

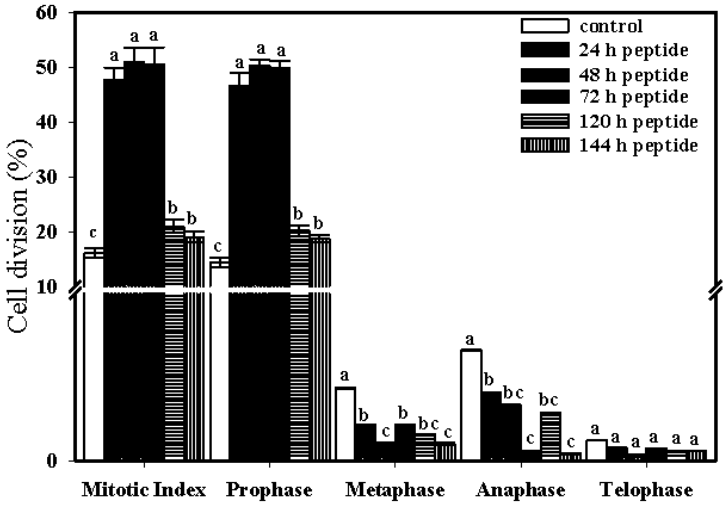

Fig3.Effect of lowmolecular weight mungbean peptides (3 kDa- 0.5 kDa)on the cell division of onion root tips*in vitro*. Values represent mean ± SE. Bars showing different letters (a-c) indicate significant differences accordingto Duncan's test at $p < 0.05$

Fig4.The identified dividing phases in onion root tips. Major phases are – prophase, metaphase, anaphase and telophase for dividing phase, and interphase as the non-dividing phase.

Isolated Mungbean Peptides from Early Hours of Germination Actively Induce the Cell Division Acting as Specific Signaling Molecules

Cell division can be defined as separation of a cell into two daughter cells. In higher eukaryotes this process involves the sequential division of the nucleus, i.e.-mitosis, first and then of the cytoplasm, i.e.-cytokinesis [34]. Cell division might be considered to be the most important phenomenon in the growth and development of any higher organisms. It is now well established that both intra- and inter-cellular signaling molecules play vital roles in controlling cell division patterns; and hormones and peptides are major among them [35]. Our present results showed that the peptide(s) isolated at 24, 48 and 72 h, but not those at 120 and 144 h of germination, induced the cell division; (Figs 3 and 4). The mitotic index (MI) was also found to be increased by 196, 216.3 and 213.4 % in onion root cells treated with the peptide(s) isolated from 24, 48 and 72 of germination as compared to controls, respectively. The increased percentage in MI was mainly due to the increment in prophase only. The other phases of mitosis, i.e. - metaphase, anaphase and telophasewere significantly inhibited by the peptide(s) treatment as compared to controls, respectively (Fig 3). To revisit cell division in the actively dividing eukaryotic cells, the 'cell cycle' comprises of two major phases, the mitotic phase and interphase [34]. During interphase, which consists of the G_1, S and G_2 phase, the cell roughly doubles its mass, synthesis of DNA, and waits for specific signals to enter into the mitotic phase; especially prophase - the initial phase of the mitotic phase. Our results indicate that the isolated peptide(s) actas signaling molecule guiding the active cell mass of meristematic zone to enter into the prophase, and induce cell division. In 1996, Y. Matsubayashi and Y. Sakagami isolated a sulfated penta-peptide, named as phytosulfokine-α (PSK), from mesophyll culture of asparagus, which promoted cell division at nanomolar concentrations even at initial cell densities as low as 320 cells mL^{-1} [8]. Researchers have also identified some other peptides, like Clavata, CLE, Polaris, etc., which also significantly affect the meristem organization and cell division in plants [5]. It is again tempting to speculate the presence of similar specific types of peptides, which execute inductive signal on the cell division process, in the isolated peptide pool from germinating mungbean seeds. However, we will need further work to provide evidence for these peptides.

Concluding Remarks

Peptides have already been recognized as major bioactive molecules, which can effectively mediate or help to mediate diverse developmental processes in plants.Even though, their synthesis is not well defined, research is progressing on the complete '*peptidome*', by isolating, identifying and characterizing the peptides therein. In the present experiment, we have isolated the total peptide contents (between 3 kDa to 0.5 kDa) from germinating mungbean seeds, and studied their dynamic appearance together with biological activity. Our data confirm that the peptides appearing at higher amount during the active germination phase (probably up to 72 h of germination) of mungbean seeds show positive control over α-amylase activity and cell division, and even mimic hormonal activity. This temporal variation in occurrence and activity might point towards a conditional synthesis of peptides in germinating seeds of mungbean. In future studies, we will attempt to identify these and other individual peptides in the peptidome of mumgbean, by applying high-throughput proteomics/ peptidomics technique.

Acknowledgement

AS gratefully acknowledges the financial support in the form of CSIR-SRF from Council of Scientific and Industrial Research (CSIR), New Delhi, Government of India; and HOD, Department of Botany, University of Calcutta, India, for providing necessary laboratory facilities during the present work.

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