Selective binding of *trans*-zeatin present in *Kappaphycus alvarezii* sap using an ionic liquid

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***trans*-Zeatin is the first cytokinin extracted from plant tissue and has been used extensively in physiology and biochemistry research of plant hormones, plant protoplast cultivation, cultivation of fused cells and breeding work, etc. This cytokinin is reported to be present in the liquid plant stimulant obtained by mechanically squeezing fresh *Kappaphycus alvarezii* seaweed. In this study, it was observed that addition of an ionic liquid, 1-butyl-3-methylimidazolium tetrafluoroborate [Bmim]BF_4, into the above plant stimulant gave instant formation of a white precipitate (KBF_4) due to ion-exchange between the imidazolium cation of the ionic liquid and potassium present in the plant stimulant. Characterizations of the precipitate employing various analytical tools confirmed presence of *trans*-zeatin in the precipitate.**

Keywords: Ionic liquid, *Kappaphycus alvarezii*, selective binding, *trans*-zeatin.

KAPPAPHYCUS alvarezii is a commercially important red seaweed growing in tropical waters and is a major source of κ-carrageenan, an important polysaccharide used extensively in food and beverage industry[1]. The sap (*K*-sap) obtained from the fresh seaweed has proven as an effective foliar spray for enhancement of yield of various crops up to 46% (refs 2–4). In order to identify the actual organic constituents responsible for the efficacy, we have reported identification and quantification of few plant growth regulators (PGRs) in the sap employing electrospray-mass spectrometry (ESI-MS)[4]. However, the biggest disadvantage of extraction of PGRs by conventional solvent extraction method is the large-scale use of volatile solvents and the tedious extraction procedures. Further, we have attempted to prepare *K*-sap formulations by selectively expelling gibberellin (GA_3) by solvent extraction and have observed positive impact on biomass productivity of *Zea mays* besides grain yield[5]. Room temperature ionic liquids (RTILs) are salts, which remain in liquid state at room temperature and are considered as a novel class of benign media alternative to the conventionally used organic solvents[6–8]. Apart from applications in other fields, in recent years, increasing applications in liquid–liquid extractions of metal ions and organic bioactive compounds from plants using ionic liquids (ILs) are documented[9,10]. ILs are used for the extraction of natural products such as alkaloids, drug molecules and biopolymers such as cellulose, lignin, etc.[11–13]. Also, there is a sole report available on extraction of indole-3-butyric acid (an auxin) from pea plants using imidazolium-based hydrophobic ionic liquids containing PF_6 and BF_4 anions[14]. However, so far the ILs are being treated as only solvent media for extraction purpose and the high polarity of the solvent is the main reason behind extraction capability of these benign solvent systems. Although the ILs are reported to have good ion-exchange capability[15], this property has not been utilized for the extraction of any chemicals present in liquid phase. In this communication, the ion-exchange property of 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim]BF_4) has been utilized for the formation of the KBF_4 precipitate by exchanging potassium ion present in the *K*-sap with the imidazolium cation of IL. FTIR, HPLC, ESI-MS and tandem mass spectrometry (ESI-MS/MS) analyses showed selective presence of *trans*-zeatin in the precipitate. In contrast to the tedious extraction procedure for *trans*-zeatin from plant extracts, the reported method is simple and less time-consuming. Also, *trans*-zeatin is the first cytokinin to be extracted from plant tissue and has important and extensive applications in physiology and biochemistry researches of plant hormones, plant protoplast cultivation, the cultivation of fused cells and breeding[16].

Fresh *K. alvarezii* was collected from the cultivation sites of the southeast coast of India at Mandapam (9.28°N 79.12°E) during April 2012. The fresh seaweed was mechanically crushed to obtain the sap (*K*-sap, Figure S1, in Supporting Information, see online)[2]. *Ulva faciata* was collected from the natural habitats in Diu (20.54°N 70.58°E), west coast of India during November 2012. Next, 440 g of fresh seaweed was ground adding 250 ml of sea water and mechanically squeezed to obtain 320 ml of sap (*U*-sap; Figure S2, see Supporting Information online).

Standard indole-3-acetic acid (IAA) ($C_{10}H_9NO_2$, MW 175.18) and GA_3 ($C_{19}H_{22}O_6$, MW 346.38) were purchased from S.D. Fine Chemicals (Mumbai). *trans*-zeatin ($C_{10}H_{13}N_5O$, MW 219.11) was purchased from Sigma-Aldrich (USA), 1-butyl-3-methylimidazolium chloride [Bmim]Cl and 1-butyl-3-methylimidazolium bromide [Bmim]Br were purchased from Merck (Germany), while 1-butyl-3-methylimidazolium hexafluorophosphate [Bmim]PF_6 and [Bmim]BF_4 were purchased from TCI Fine Chemicals, Japan. All chemicals were used as received without further purification.

Two hundred microlitres of IL was added separately in 500 µl of *K*-sap and *U*-sap at room temperature (Figure 1 and Figure S2 in Supporting Information, see online). The precipitate formed in the case of [Bmim]BF_4 was separated by centrifugation followed by thorough

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Figure 1. Precipitation in *Kappaphycus alvarezii* sap (*K*-sap) upon addition of 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim]BF_4) and identification of *trans*-zeatin in the isolated precipitate.

washing with deionized water and dissolution in 100 µl of dimethyl sulphoxide (DMSO) and finally 100 µl of *n*-butanol was added. The solution was filtered with 0.2 µl nylon filter and used for HPLC and mass spectrometry experiments.

Cytokinins were extracted from *K*-sap using 25 ml of sap[4]. For the extraction of cytokinins, the pH of the sap was adjusted to 3 by dropwise adding of 1N HCl followed by extraction with dichloromethane (DCM) (×3), which was collected and stored. The pH of the aqueous layer was adjusted to 8 with 1N NaOH, followed by extraction with *n*-butanol (×3). The DCM and *n*-butanol layers were evaporated to dryness, the residue was dissolved in minimum amounts of *n*-butanol and mixed together.

The dry samples were dispersed in acetone and coated on aluminum stubs and evaporated to dryness followed by recording of their SEM images on a LEO 1430 VP instrument employing accelerating voltage of 18 kV. FTIR analyses were recorded on a Perkin Elmer Spectrum GX (FTIR System, USA) by taking 2.0 mg of sample in 600 mg of KBr. All spectra were average of two counts with 10 scans each and a resolution of 5 cm^{-1}. The optical micrographs were recorded on an optical light microscope (Fine Vision, India) at 100× magnification.

The HPLC column used was Enable C18H 5 µm 150 mm × 4.6 mm packed with C18 stationary phase of 5 µm particle size (Spincotech, India). The HPLC solvent system comprised of A: water and B: methanol and 0.1% formic acid was added in both the solvents. The elution was started with 30% B from 0 to 2 min, increased linearly to 100% B from 2 to 20 min and held for 2 min and finally decreased linearly to 30% B in 22 to 25 min. The solvent flow rate was kept at 0.8 ml/min throughout the experiment. Column heater temperature was maintained at 40°C and UV detection was carried out at 254 nm (ref. 17).

ESI-MS measurements were carried out on a Q-TOF micro mass spectrometer (USA), equipped with an electrospray ionization source, time-of-flight (TOF) analyser and micro-channel plate (MCP) detector. Standard *trans*-zeatin was dissolved in *n*-butanol. The concentration was maintained at 10 ppm for the optimization studies. The mass spectrometer was run employing direct infusion technique (DIMS). Mass fragmentation patterns were recorded in ESI-positive mode (ESI^+). Parameters such as desolvation temperature (150°C), source temperature (90°C), syringe rate (10 µl/min), ion energy (2.0 V) and collision energy (7.0 V) were maintained constant for all ESI-MS measurements, whereas capillary voltage and sample cone voltage were optimized for each sample. Nitrogen gas was used as a nebulizer gas to accelerate the droplets of the samples formed during electrospray at a flow rate of 450 l/h. Mass spectra were recorded in the mass range of *m*/*z* 50–500 and data were processed using Masslynx 4.0 software (Waters Corp., UK). For MS/MS studies, the parameters were once again optimized to obtain proper mass fragmentation.

K-sap with 75% w/w could be obtained from fresh *K. alvarezii* by mechanical squeezing. Fresh *Ulva fasciata*, a green seaweed gave only 46% w/w sap upon mechanical squeezing on addition of seawater (*U*-sap; see Figures S1 and S2 in Supporting Information online). Next, 0.2 ml of different ILs having 1-butyl-3-methylimidazolium as common cation, but different anions such as hexafluorophosphate (PF_6^-), bromide (Br^-), chloride (Cl^-) and tetrafluoroborate (BF_4^-) were added into 500 µl of *K*-sap and *U*-sap separately. Instant formation of white precipitate (within 5s) was observed in the case of [Bmim]BF_4 to *K*-sap (Figure 1). However, the IL did not result in the formation of any precipitate upon addition into *U*-sap (Figure S2 in Supporting Information, see online). The other ILs used in this study did not result in the formation of any precipitate upon addition into the above saps (data not shown).

The *K*-sap is reported to contain about 4% w/v of KCl (ref. 18) along with other micro and macronutrients and the sap used in this study contained about 3.36% potassium (Table S1 in Supporting Information online). On the other hand, *U*-sap used in this study had only 0.045% w/v of potassium. The formation of white precipitate in the former sap was favoured due to the presence of higher amount of K^+, which took part in ion exchange with imidazolium cation of IL (in this case the ionic product was

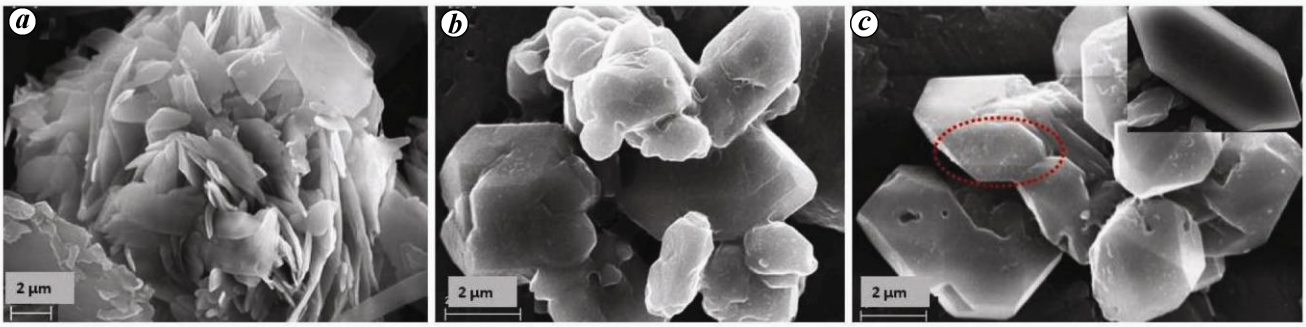

Figure 2. SEM image of (***a***) pristine *trans*-zeatin, (***b***) KBF_4 precipitate obtained after addition of [Bmim]BF_4 into 4% w/v KCl solution and (***c***) KBF_4 precipitate obtained after addition of [Bmim]BF_4 to *K*-sap.

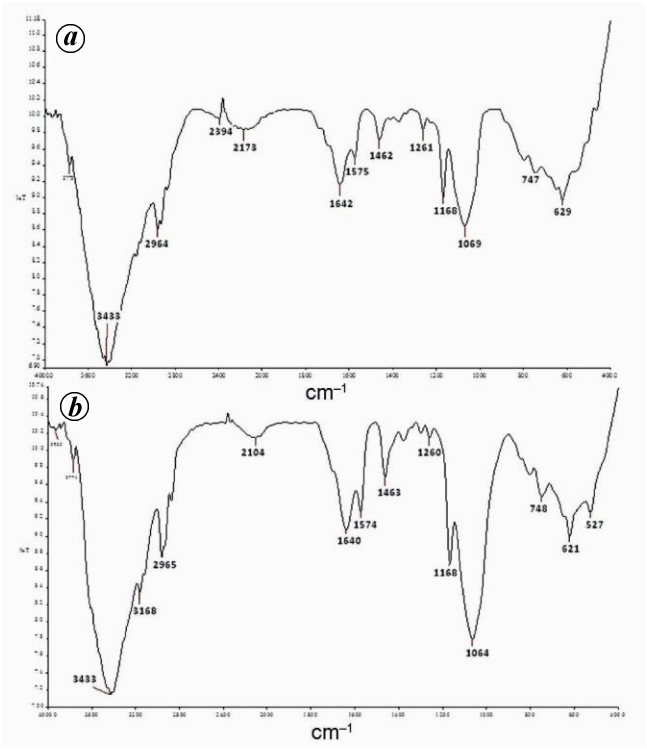

Figure 3. FTIR spectra of (***a***) standard *trans*-zeatin and (***b***) KBF_4 precipitate obtained after addition of [Bmim]BF_4 to *K*-sap.

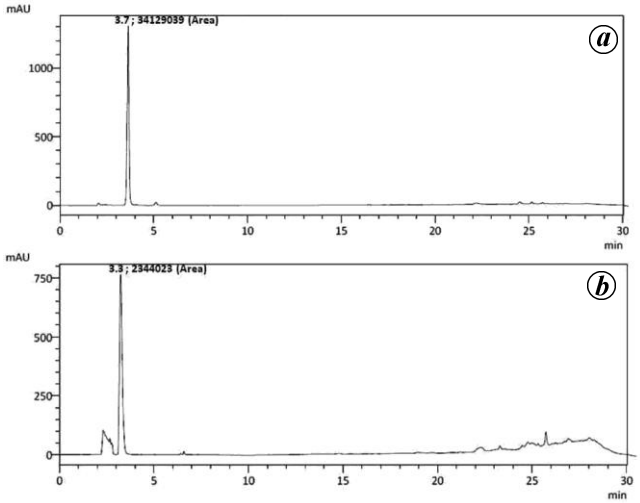

Figure 4. HPLC spectra of (***a***) standard *trans*-zeatin and (***b***) organic extract of KBF_4 precipitate obtained after addition of [Bmim]BF_4 to *K*-sap.

more than the solubility product). In the case of the latter sap the ionic concentration (product of the concentrations of K^+ and BF_4^-) was less than the solubility product of the complex and hence precipitation did not take place. The phenomenon could be reconstituted upon addition of IL in 4% KCl solution (simulated for *K*-sap), where formation of white precipitate was observed, while addition of the same IL into 0.04% KCl and 0.04% NaCl solution (simulated for *U*-sap) did not result in the formation of the precipitates (Figure S3 in Supporting Information online). After the ion exchange, [Bmim]Cl was formed inside the sap, toxicity of the ILs is a matter of concern[19] and [Bmim]Cl is reported to be toxic to animals upon very high levels of administration[20]. There are no reports available on toxicity of the ILs to plants or humans. If 100% ion exchange of ILs is considered, the amount of [Bmim]Cl expected to be formed is 306 mg/l in the sap. This is a much lower concentration expected to be non-toxic for applications on plants. The SEM images of the precipitate showed presence of ordered morphology with a few white particles on the surface (Figure 2 *a*–*c*), indicating adsorption of some organic compound on the surface. However, no such white particles were formed on the surface of the precipitate obtained from the simulated KCl solution (Figure 2 *b*). The SEM–EDX indicated formation of KBF_4 with organic compound having carbon, nitrogen and oxygen (ESI, Figure S4 in Supporting Information online). Formation of KBF_4 was further confirmed from the mass fragmentation pattern, where *m*/*z* of 165.06 due to (KBF_4 + K) adduct was observed (Figure S5 in Supporting Information online).

The FTIR spectra of the precipitate were recorded and compared with those of standard plant growth hormones present in the sap such as IAA, *trans*-zeatin, kinetin and GA_3. The spectra matched well with those of pristine *trans*-zeatin (Figure 3 *a*). For standard *trans*-zeatin the band at 1642 cm^{-1} is typical for C=N stretching vibration of purine ring[21]. The N_9-H stretching showed the band at 1462 cm^{-1} (ref. 22). Apart from these characteristic

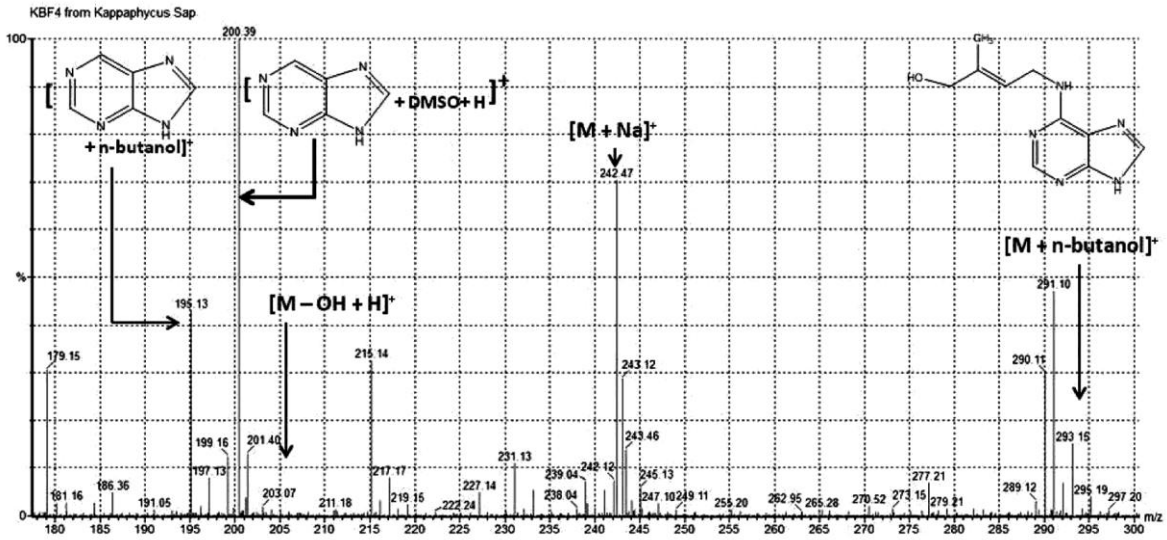

Figure 5. Mass fragmentation for *trans*-zeatin present in the KBF_4 precipitate obtained after addition of [Bmim]BF_4 into *K*-sap.

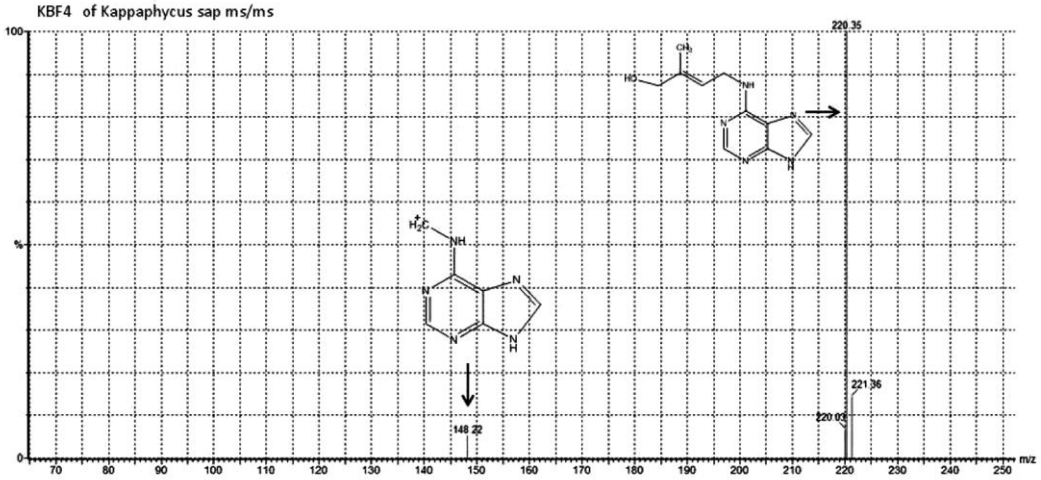

Figure 6. Tandem mass fragmentation for *m*/*z* 220 of *trans*-zeatin present in the KBF_4 precipitate obtained after the addition of [Bmim]BF_4 to *K*-sap.

bands, pristine *trans*-zeatin showed stretching vibrational bands due to N–H and C–N at 3433 and 1260 cm^{-1} respectively. The KBF_4 precipitate also showed similar bands (Figure 3 *b*) indicating the presence of *trans*-zeatin in the precipitate.

Various solvents such as acetone, dichloromethane, DMF, DMSO, acetonitrile, etc. were used to dissolve the KBF_4 precipitate. Among these, only DMSO and DMF were found to be capable of dissolving the precipitate. The precipitate was washed with water followed by dissolution in DMSO and *n*-butanol was added to it (since *n*-butanol is known to dissolve cytokinins). The mixture was filtered using 0.2 µm nylon filter paper and subjected to HPLC analyses. The standard *trans*-zeatin in *n*-butanol showed a sharp peak at 3.7 min and the sample showed a peak at retention time (RT) of 3.3 min (Figure 4). The comparable RT for the sample with pristine *trans*-zeatin gave a positive indication for the presence of *trans*-zeatin in the sample prepared from the precipitate. The HPLC profile of other plant growth regulators such as IAA, kinetin and GA_3 present in the sap is shown in the Supporting Information online (Figure S6). The adduct formation of *trans*-zeatin with KBF_4 was further confirmed from the appearance of mass fragmentation at *m*/*z* of 419.18 due to (KBF_4 + *trans*-zeatin) adduct (Figure S7, see Supporting Information online). The *trans*-zeatin molecule after fragmentation formed adduct with KBF_4 and potassium as evident from the *m*/*z* at 365.51 (Figure S7, see Supporting Information online). The quantification of *trans*-zeatin based on the peak area of liquid chromatograms showed 19.8 ± 0.72 ppm in the precipitate (Supporting Information online). The *trans*-zeatin extracted by conventional method and estimated by HPLC was found to be 26.5 ± 2.3 ppm. In the present case a dis-

tinct peak due to *trans*-zeatin was observed in the HPLC profile, indicating selective presence of the same. The quantification of zeatin shows presence of 74.71% of the total zeatin in the KBF_4 precipitate.

The same sample used for HPLC was analysed by ESI-MS employing direct infusion technique (DIMS). The mass fragmentation at *m*/*z* 242.47 was due to the adduct (*trans*-zeatin + Na^+). Appearance of *m*/*z* 203.07 was due to the fragmentation of *trans*-zeatin (Figure 5). The adduct formation of purine base of *trans*-zeatin with *n*-butanol and DMSO is evident from the *m*/*z* at 195.13 and 200.39 respectively.

The MS/MS fragmentation at *m*/*z* 220 of the organic extract (Figure 5) resulted in the formation of daughter ion peak at *m*/*z* 148.22 (Figure 6). This is similar to the fragmentation pattern reported for standard *trans*-zeatin[4,23]. The above mass fragmentation and the tandem mass spectroscopic fragmentation confirmed the presence of *trans*-zeatin in the precipitate. Moreover, boron is reported to interact with cytokinins and zeatin is reported to associate through π–π interactions[22,24]. Perhaps, these interactions are responsible for the selective binding of *trans*-zeatin with KBF_4.

We have described a new strategy to extract selectively *trans*-zeatin (about 75%) from *K*-sap using an ionic liquid. The precipitate formed upon addition of 1-butyl-3-methylimidazolium tetrafluoroborate into the sap due to ion exchange between imidazolium cation of the ionic liquid and potassium present in the sap found to selectively bind to *trans*-zeatin present in the sap. The presence of the cytokinin was confirmed using multiple analytical techniques. The interaction between boron of KBF_4 and *trans*-zeatin and further association of zeatin molecules through π–π interactions are perhaps responsible for the selective binding of the cytokinin to the precipitate.

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