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Bio-physical evaluation and *in vivo* delivery of plant proteinase inhibitor immobilized on silica nanospheres

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ABSTRACT

Recombinant expression of *Capsicum annuum* proteinase inhibitors (CanPI-13) and its application *via* synthetic carrier for the crop protection is the prime objective of our study. Herein, we explored proteinase inhibitor peptide immobilization on silica based nanospheres and rods followed by its pH mediated release *in vitro* and *in vivo*. Initial studies suggested silica nanospheres to be a suitable candidate for peptide immobilization. Furthermore, the interactions were characterized biophysically to ascertain their conformational stability and biological activity. Interestingly, bioactive peptide loading at acidic pH on nanospheres was found to be 62% and showed 56% of peptide release at pH 10, simulating gut milieu of the target pest *Helicoverpa armigera*. Additionally, *in vivo* study demonstrated significant reduction in insect body mass (158 mg) as compared to the control insects (265 mg) on 8th day after feeding with CanPI-13 based silica nanospheres. The study confirms that peptide immobilized silica nanosphere is capable of affecting overall growth and development of the feeding insects, which is known to hamper fecundity and fertility of the insects. Our study illustrates the utility and development of peptide-nanocarrier based platform in delivering diverse biologically active complexes specific to gut pH of *H. armigera*.

1. Introduction

Delivery of bio-molecules often entails both biological and synthetic milieu for their release at the necessary site of action [1–3]. Bio-delivery of protein/peptide(s) owing to their magnificently diverse and remarkable structural and functional properties is of primary interest. These features are governed by their structural conformation which in turn is tailored by surrounding environment such as molecular crowding, protonation, temperature, pH, *etc.* [4–6]. Small peptides, owing to their multi-level function in biological system are produced recombinantly or chemically synthesized for their broad spectrum application in the form of biomedical drugs [7], signalling, [8] inhibitors of specific enzyme targets [9] and many more. Thus, efficient application of protein/peptide(s) in the non-native environment depends on the designing of delivery carrier without altering the function in achieving competent targeted emancipation. To achieve this, nanoparticles (NPs) have been considered as one of the potential system for targeted delivery in the desired environment [10]. Choice of NPs of varied sizes and structures depends on its specific application. Specifically, NPs have long been known for their drug delivery applications. Several studies have investigated the interaction of NPs with complex set of proteins such as serum proteins. In addition, due to their physico-chemical features, such as chemical composition, surface functionalization, shape, angle of curvature, surface charges, *etc.* [11,12]. NPs adapts to the physiological environment by forming protein 'corona' through adsorption thereby attaining biological identity in the system [13,14]. Engineered particles can be utilized as a potential platform to develop specific protein 'corona' *in vitro*. It can thus deliver target molecule to desired site in order to enhance their applicability in specific environment [15]. Towards this, biocompatible silica based nanomaterials which possess unprecedented advantages as drug delivery nanocarriers have been structurally and chemically modified for their successful explorations [16–18].

In this study, we explored silica nanospheres (SiO_2N) and Santa Barbara Amorphous (SBA-15) for the maximum loading of proteinase inhibitor (PI) peptide and its stability. Application of

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recombinantly expressed PIs which was sourced from insect non-host plant, *Capsicum annuum*, may prove to be a potential system for providing protection from Lepidopteran insects to limit the crop damage [19]. In the initial screening we noted SiO_2N to be the promising material. Hence, we endeavoured to use silica-based nanocarrier with PI peptide for their potential use in agriculture implications. Towards this, silica nanospheres (SiO_2N) offer physical and chemical tunability with resourceful usage as a prudent bioactive nanocarrier [16]. Also, large pore diameter and high surface area of self assembled silica particles provides platform for immobilization of many bioactive molecules imparting additional advantage of sturdiness and durability. Furthermore, it is prerequisite to protect the stability of protein/peptide in the outer environment from chemical and physical parameters such as temperature, moisture, plant's chemical secretions, soil pH, composition, *etc.* [20]. Thus, we aimed to use biocompatible SiO_2N–PI duo complex, firstly to avoid such constraints [19] and secondly to allow inhibitor molecule to reach and maintain function in the gut of insect. In addition, the comprehensive mapping of the nano-bio interface following immobilization is anticipated along with its effect *in vivo*. Exceptionally, *in vivo* delivery of SiO_2N–PI is unconventional over to the traditional acidic milieu delivery below 5.5 pH. To our knowledge, this is the first report to establish alkaline pH mediated PI-peptide release from charged silica solid surface to develop an effective biopesticide for crop protection.

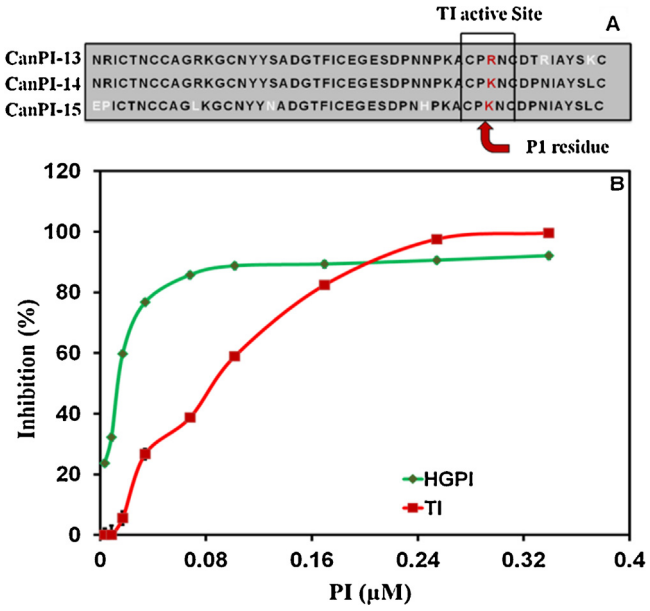

Fig. 1. (A) Sequence comparison of CanPI-13 with CanPI-14 and 15, highlighting conserved trypsin inhibitory active site along with presence of lysine (K) and Arginine (R) residue; other sequence variations are shown in white colour and (B) proteinase inhibitory activity against bovine trypsin and *H. armigera* gut protease mixture (HGP) was measured at increasing concentration. Standard error bars are shown for $n = 3$.

2. Results and discussion

2.1. Characterization of CanPI-13

PIs are natural defensive proteins found in the plants and induced upon insect attack/damage in local and systemic tissues [21]. *C. annuum* PI (CanPI-13) is a small peptide of around 5.9 kDa and is one of the variant amongst series of inhibitors, belonging to wound inducible Pin-II PIs confined to Solanaceae family [21,22]. PI was expressed in yeast (*Pichia pastoris*) system to obtain recombinant inhibitor protein. Multiple sequence alignment of PI and its variants revealed that it consists of single functional domain which determines its specificity against trypsin-like serine proteases as evident by the appearance of conserved Lysine (K) or Arginine (R) in the sequence at reactive P1 residue. Furthermore, the presence of eight cysteine residues in the sequence will lead to the formation of four disulphide bonds imparting structural stability to PI molecule (Fig. 1A). Activity of recombinant CanPI-13 was validated against commercially available bovine trypsin and gut extracted trypsin(s) of *Helicoverpa armigera* larvae exhibiting maximum 100% and 91% inhibition, respectively (Fig. 1B). PI inhibitory potency towards insect trypsin is supported by its interference with the protein digestion leading to arresting the growth, development and hamper fecundity and fertility of pest *H. armigera* [19]. Importantly, by virtue of this inhibitor peptide, which exhibits more activity towards cocktail of insect (*H. armigera*) trypsins (IC_{50} 0.01 μM) than bovine trypsin (IC_{50} 0.09 μM), is potentially advantageous for their insecticide use.

2.2. Immobilization of CanPI-13 and biophysical evaluation

In initial study, SiO_2N and SBA-15 were chosen to compare the loading capacity of recombinant CanPI-13. SBA-15 was synthesized as per reported procedure [23]. It was found that the Brunauer, Emmett and Teller (BET) N_2 adsorption surface area 902 m^2/g with Barret–Joyner–Halenda (BJH) adsorption cumulative volume of pores 0.98 cm^3/g and BJH adsorption average pore having width (4 V/A) = 90.2 Å. Whereas, Stöber method was followed to synthesize SiO_2N by hydrolyzing tetraethyl orthosilicate (TEOS) in highly basic media [24,25]. The surface area and hydroxyl group density of obtained SiO_2N was increased by mild etching in aqueous KOH solution. Furthermore, BET surface area was found to be 13 and 25 m^2/g with BJH adsorption cumulative volume of pores 0.066757, 0.212843 cm^3/g and BJH adsorption average pore having width (4 V/A) = 411.892 and 569.993 Å. The ^{29}Si NMR (nuclear magnetic resonance) analysis showed increase in Q^3 species (−100 ppm) than the parent SiO_2N, which indicated increase in hydroxyl density on the SiO_2N surface (ESI Fig. S1-A and B). At first, 1 mg of PI was incubated with 10 mg SBA-15 and SiO_2N at pH 4.0. CanPI-13 immobilization on silica was carried out by considering peptide's isoelectric point (pI = 6.2), which upon varying the solution pH below and above pI endowed net positive/negative charge on rCanPI-13 due to gain or loss of protons (H^+). However, equal amount of rCanPI-13 was estimated to be adsorbed on SiO_2N despite of the presence of large surface area of SBA-15 as compared to SiO_2N (ESI Fig. S2†). Zeta potential measurements revealed the surface charges on silica nanostructures and native protein as observed at different pH. SBA-15 carried almost neutral surface charge whereas, SiO_2N displayed negative charge (ESI Table 1†). The study revealed that protein adsorption on the particle surfaces rely majorly on electrostatic interaction and protein flexibility to access nano-surface and is further regulated by specific amino acid residues [26]. Thus, rCanPI-13 was incubated with negatively charged SiO_2N and immobilization was found to be occurring at pH 4 with negligible adsorption at alkaline pH 7 (ESI Fig. S3-A†). This is because of the positive mean zeta potential (2.16 mV) of CanPI-13 at pH 4 as compared to negative zeta potential (−10.36 mV) displayed at higher pH. Nevertheless, zeta potential of SiO_2N–PI showed positive charge showing 16.82 mV at pH 4 (Fig. 2A) which clearly stated the dominance of positively charged PI peptide on silica surface. Also, the formation of PI mono or multi layers on SiO_2N surface substantiates the SiO_2N–PI protonation occurring at pH 4 as corroborated in previous studies [27,28]. These SiO_2N were observed to be mono dispersed (Fig. 2B and C) with size of ∼240 nm (ESI Fig. S3-B†) demonstrated by scanning electron microscopy (SEM). SBA-15 was also visualized for their morphology with and without PI