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Protein chips for detection of mite allergens using Kunitz-type protease inhibitors.

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Abbreviations: Kunitz-type protease inhibitor (KPI); Soybean Bowman-Birk protease inhibitor (S-BBI).

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Abstract

Stored food and house dust arthropods include many species of mites and beetles affecting human health. For diagnostic tests proteases such as trypsin are utilized as they are indicators of the presence of allergen contaminants in food. We recently characterized Kunitz-type protease inhibitors (KPIs) from *Solanum palustre*. Here we studied biotechnological applications of KPI-B1 and B4. We manufactured a protein chip with immobilized KPI-B1 and B4 and showed trypsin/chymotrypsin-binding specificity indicating that the recombinant proteins have protease selectivity. We employed the protein chip to capture mite proteins belonging to the protease family with polyclonal anti-mite antibodies. The mite diagnostic chip can be useful for detecting mite allergens.

Introduction

Kunitz-type protease inhibitors (KPIs), with size 22-24 kDa, are a class of plant protease inhibitors that play an important role in plant defence against pathogens and insects [1]. The Kunitz-type inhibitors found in potato and other *Solanum* species are divided into three homology groups, A, B and C. KPI-A group includes inhibitors of aspartic proteases such as cathepsin D [2, 3]. Group KPI-B contains inhibitors of serine proteases [2, 3], e.g., trypsin and chymotrypsin. KPI-C group includes inhibitors of plant cysteine proteases as well as some non-protease hydrolytic enzymes such as α-amylase [4].

In a previous work, we characterized the inhibitory activity of KPI group B proteins [3]. KPI-B1 and B4 were the most active inhibitors of trypsin at nanomolar concentration (84.8 nM for B4 and 345.5 nM for KPI-B1). Recombinant KPI-B proteins were tested on a panel of model proteases isolated from vertebrates, invertebrates and fungi. The assays revealed that the protease inhibitory activity is not only limited to bovine trypsin but also to red king crab (*Paralithodes camtschaticus)* trypsin which is structurally close to trypsin from beetle and butterfly larvae (data not shown). Thus, KPI groups contain protease inhibitors with large feasibility to trypsins from different origins.

In the present work, recombinant KPI-B1 and B4 were tested together with a known protease inhibitor in a protein chip to study the interaction with trypsin, and, together with two KPIs of group A, the interaction with three different proteases.

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Mites and beetles are often found in contaminated stored products and farming environments. The contamination of stored food by arthropods may seriously endanger human health [5], as they can cause allergies [6-8] and serve as vectors of fungal allergens and mycotoxins producing fungi [9]. More than twenty classes of major allergens are caused by house dust or stored product mites (Table 1). Six of these allergens are proteases and related hydrolytic enzymes [5-8]. Mites and beetles contain digestive enzymes including serine-type proteases (chymotrypsin, trypsin and a collagenolytic protease), a cysteine protease and an aspartic protease, and different proteases are abundantly present in mites' faeces.

Diagnostic tests have been developed with fixed antibodies on strips or microplates as capture probes to detect allergens, i.e., using trypsin to detect soybean allergens as STI, a marker of the presence of soybean contaminants in food [10, 11]. The KPI chips were tested in the detection of mite proteins supposed to be proteases. Protein visualization anti-mite antibodies allows relative quantification of mite proteases bound to each KPI group in each individual experiment, but is not feasible for comparison between different experiments, due to the laser scanner used.

The ultimate goal is the application of KPI proteins in the detection of mite hazards. The miniaturization of these assays will enable the creation of high-throughput tools and new diagnostics.

2. Materials and Methods

2.1. Proteins used in this work

Recombinant protein production and purification were carried out as previously described [3] and checked by western blot using a polyclonal antibody against a potato KPI-B10 protein (kindly provided by Dr. A. Shevelev). Four His-tagged KPIs, two of group B, KPI-B1 (AY945740) and KPI-B4 (AY945743), and two of group A, KPI-A1 (FJ969213) and KPI-A2 (FJ969214), were produced and used for subsequent biotechnological characterizations. Proteases (trypsin, chymotrypsin and cathepsin B), the protein controls and reagents were from Sigma (Sigma-Aldrich, Milwaukee, WI, USA).

2.2. Protein chip preparation

All recombinant protease inhibitors were desalted with Zeba desalt spin columns (Pierce, Rockford, IL, USA). The Kunitz-type protease inhibitors and the positive control, soybean Bowman-Birk inhibitor, were prepared at different concentrations (0.1, 0.2, and 0.4 mg/ml) in a $NaHCO_3$ buffer (0.1 M, pH 9) with glycerol (40% w/v) and arrayed on epoxysilane-coated Nexterion slides (Schott,

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Jena, Germany) with a robotic printing SpotArray 24 system (Perkin Elmer, Waltham, MA, USA). The SpotArray 24 was employed to print in the miniaturized protein chips. Each spot printed with SpotArray 24 contained 50-70 nl of 0.1, 0.2 and 0.4 ng/nl KPI. The printing was performed in triplicate for each protein at 4° C and about 60% humidity using to the SpotArray humidity control. The printed slides were incubated at room temperature for two hours at 60% humidity in a dark room to allow the reaction between epoxy groups and water. Then, the printed slides were kept dried in the dark for later use, or immediately used. The protein chips ready for use were incubated for one hour on a shaker at room temperature in a phosphate-buffered saline (PBS) containing glycine (0.5 M) and bovine serum albumin (BSA; 1% w/v) to block non-specific binding. After that, the slides were washed with PBS + BSA (0.1% w/v) and dried in a centrifuge at 500 g x 2 min.

2.3. Protein chip assays

Each protease (trypsin, chymotrypsin and cathepsin B) was labelled with an Alexa-555 protein labelling kit (Molecular Probes, Life Technologies, Carlsbad, CA, USA) and purified with gel-filtration biogel P-6 (Bio-Rad, Hercules, CA, USA) to remove free dye according to the manufacturer's protocols. The probe was then applied as follows: 50 µl of labelled, purified protease (1 mg/ml of trypsin and chymotrypsin in Tris-hydroxymethylamonomethane (TRIS) buffer 0.1 M, pH 7.5; and 1 mg/ml cathepsin B dissolved in phosphate buffer 0.1 M, pH 6.5) was applied with the hybridization buffer onto the slide, using the coverslip method [12-14] and incubated for one hour in the dark at room temperature in a humidity chamber (70% humidity). After incubation, the slides were washed with PBS containing Tween-20 (0.2% w/v) and BSA (0.1% w/v) for 15 minutes on a shaker at 4° C. Next, the slides were washed twice with PBS + BSA (0.05% w/v) for five minutes on a shaker at 4° C and dried in a centrifuge at 500 g x 2 min. Then, the slide was scanned with a Laser Scanner 428 (Affymetrix, CA, USA).

In the detection of mite extracts, protease inhibitors (KPI-A1, KPI-A2, KPI-B1, KPI-B4 and S-BBI) were spotted on slides at serial dilutions, 0.1, 0.2 and 0.4 ng/nl using the manual MicroCaster (Whatman, Brentford, UK) producing spots with greater size, around 500 nl. After protein immobilisation and slide desiccation, the chips were used the following day.

2.4. Preparation of mite/insect allergenic proteases and detection on the protease inhibitor chip

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Mite cultures (*Acarus siro*, *Aleuroglyphus ovatus, Lepidoglyphus destructor*, *Tyrophagus putrescentiae*), and red flour beetle (*Tribolium castaneum*) culture and rabbit immunization for producing polyclonal antibodies were carried out at VURV in Prague. Protein extracts of frozen adults were obtained from starved, washed mites. Frozen adults were weighed and homogenised in phosphate buffer saline (PBS, 20 mM Na_2HPO_4, 20 mM NaH_2PO_4, 150 mM NaCl, pH 7) and 0.1% Triton X-100. A protein concentration was determined using the Bradford method (Bio-Rad, USA). Protein concentrations were: *A. ovatus* extract, 3,5 µg/µl; *A. siro* extract, 2,9 µg/µl; *L. destructor* extract, 3.2 µg/µl; *T. putrescentiae* extract, 2,8 µg/µl; and *T. castaneum* extract, 2 µg/µl. The protein extract (2-2.5 µg/µl) was diluted with PBS. Twenty µl of hybridization solution, containing one µl of 1:10 dilution, was used, so that final concentration of proteins was between 0.2 and 0.3 µg/µl. The slides were hybridised under dark, at room temperature on a microplate shaker, then washed with TRIS buffer saline pH 7.5 plus 0.1% Tween-20 (TBS-T). To visualize protease inhibitor-mite protein interactions, the chips were incubated with species-specific polyclonal antibodies, diluted 1:20 (1 µg/ml). After removal of aspecific interactions with TBS-T washes, the slides were incubated with Alexa-555-labelled protein A for the antibody detection (Figure 3). Slides were analysed with a 428 Laser Scanner (Affymetrix, USA), and images were visualised with ScanAnalyse. The sensitivity of antigen detection on protein chips was compared to results obtained using dot blot and sandwitch ELISA assays probed with the same polyclonal antibodies [15-17].

3. Results and discussion

3.1. Characterization of the protein chip

Protein chips were tested comparing trypsin binding to S-BBI, KPI-B1 and KPI-B4 recombinant proteins [4] in order to verify their performance in conditions of limited movement and orientation constrains (figure 1), Miniaturization of the protein chip was achieved using a robotic spot-arrayer, depositing 70 nl volumes on the glass. KPI–B1 and B4 at serial dilutions showed affinity for trypsin (10 ng/µl) even at 0.1 ng/nl (Figure 1). High KPI-B1 sensitivity for trypsin was put in relation to a higher stability/freedom of movement of KPI-B1 bound to the glass as compared to KPI-B4, since KPI-B1 *K*i is higher (345.5 for KPI-B1 and 84.8 for KPI-B4). This result shows the feasibility of KPI-B1 use in biotechnological applications.

The stability of KPI protein chips was investigated. A set of printed slides was stored for one month at 4° C and their performance was tested. No evident differences were detected

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compared to a freshly-printed chip. KPI proteinase inhibitors were stable for a long time without loss of activity.

3.2. Differential KPI binding and protease specificity

KPI-B1 and KPI-B4, together with two other Kunitz-type protease inhibitors (KPI-A1 and A2) were used on KPI protein chips to investigate different binding specificity of trypsin, chymotrypsin and cathepsin B (Figure 2A, B, and C). The protein chips showed that KPI-B1, KPI-B4 and S-BBI as positive control (Figure 2A) were good binding partners for trypsin, while KPI-A1 partially interacted with trypsin. The analysis of chymotrypsin binding (Figure 2B) showed fluorescent signals only for serine protease inhibitors, KPI-B1 and KPI-B4 (as previously described using in vitro assay) [4], and S-BBI. The cathepsin-B analysis (Fig. 2C) indicated that recombinant KPI-A1 partially bound to cathepsin B, while Lactoferrin, with an affinity for cathepsin B, shows the strongest fluorescence signal.

3.3. Anti-mite protein chips. Detection of mite allergen candidate proteins

In this work the application of KPIs to detect mite [15, 16] or insect [17] proteases as potential allergens [18, 19] was proposed. Protein chips were used for capturing mite candidate proteases, detected with polyclonal antibodies specific to mite species [15-17] i.e. *A. ovatus*, *A. siro*, *L. destructor*, *T. putrescentiae* and *T. castaneum*, respectively (figures 3A, 3B, 3C, 3D, 3E). Optimum resolution shown in figure 3 was achieved with 1:10 extract dilution, corresponding to 0.2 µg of total proteins, but positive results were obtained even with extract dilutions as low as 1:20 (corresponding to 100 ng of total proteins, not shown). Faint signals present in positive control might have been caused either by the manual spotting or by very low amounts of trypsin-like proteases in the sample. Possible errors in the transfer of the samples from the microplates to the glass slide could be overcome using the Robotic SpotArray. Total protein extracts (1.5 mg/ml) did not produce good signals, probably due to the presence of lipids, polysaccharides and chitins that may block the protein interactions on the chip.

In the protein chip method, the sensitivity of the assay was higher, compared to the currently used sandwich-ELISA method [15]. The detection limit of the protein chip method was 0,1 µg of total mite extract, while the detection limit for sandwitch ELISA method is 1 µg of mite proteins using 1 ml solutions. An advantage of the proposed method is the limited hybridisation volume with lower amounts of proteins, due to the very small surface of protein chips. However, considering that

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proteases represent only a small fraction of body proteins, assuming this to be lesser than one hundredth of total proteins in mite bodies, the sensitivity of the chip method is one thousand times higher. This can be explained by the high background caused by antibody binding to membranes, whereas the glass slide showed low signal-to-noise ratios.

4. Concluding Remarks

In this work, the protein chip was used to detect mite proteins. Using this tool, KPI-B1 and B4 were capable of screening mite proteases that could represent potential allergens. Presently protein chips are produced only for the determination of reactivity of IgEs toward known antigens and allergens. The development of KPI chips and assays in solution as lateral flow tests (LFT) will allow the detection of mite contamination in stored food products and in environment.

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Figure captions

Figure 1. Miniaturized protein chips in detection of fluorescent trypsin. Upper lane: KPI-B1 (left), KPI-B4 (right). Lower lane: BSA, S-BBI.

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Figure 2. Detection of three fluorescent proteases on KPI chips. Protein concentrations: 0.1, 0.2 and 0.4 ng/nl (left to right).

2-A. Selective binding of trypsin.

2-B. Selective binding of Chymotrypsin

2-C. Selective binding of cathepsin B

2-D. A scheme of spotted proteins. Upper lane: KPI-A1, KPI-A2 and KPI-B1
Lower lane: negative control (BSA), KPI-B4, positive control (S-BBI in 2A, 2B; Lactoferrin in 2C)

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Figure 3.

Detection of mite proteases using KPI chips.

3A. Detection of *Aleuroglyphus ovatus* using anti-*A. ovatus* antibodies

3B. Detection of *Acarus siro* using anti-*A. siro* antibodies

3C. Detection of *Lepidoglyphus destructor* using anti-*L. destructor* antibodies

3D. Detection of *Tyrophagus putrescentiae* using anti-*T. putrescentiae* antibodies

3E. Detection of *Tribolium castaneum*. using anti-*T. castaneum* antibodies

3F Scheme of protein spots (0,4 ng/nl, upper lane; 0,2 ng/nl, middle lane; 0,1 ng/nl, lower lane).

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Table 1: The major allergen classes in house dust mites and storage product mites [20, 21] according to websites and to the International Union of Immunological Societies' (IUIS) rules for allergen nomenclatures.

Legends: ??? unknown function

+ suggested allergens according to their amino acid sequences and homology to known allergens

• enzymatic activity proved

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Class	Biochemical function	Stored product species					Dust species		
		Acarus siro	*Glyciphagus domesticus*	*Lepidoglyphus destructor*	*Tyrophagus putrescentiae*	*Aleuroglyphus ovatus*	*Blomia Tropicalis*	*Dermatoph. farinae*	*Derrmatoph. Pteronyssinus*
1	Cysteine protease				+	+	Blo t 1	Der f 1	Der p 1
2	NPC2 family		Gly d 2	Lep d 2	Tyr p 2		Blo t 2	Der f 2	Der p 2
3	Trypsin	+		+	+	+	Blo t 3	Der f 3	Der p 3
4	A-amylase	•		•	•	•	Blo t 4	•	Der p 4
5	???			Lep d 5			Blo t 5		Der p 5
6	Chymotrypsin	•		•	•	•	Blo t 6	Der f 6	Der p 6
7	???			Lep d 7				Der f 7	Der p 7
8	Glutathione S-transferase								Der p 8
9	Collagenolytic serine prot.	•		•	•	•		•	Der p 9
10	Tropomyosin			Lep d 10	Tyr p 10		Blo t 10	Der f 10	Der p 10
11	Paramyosin						Blo t 11	Der f 11	Der p 11
12	???						Blo t 12		
13	Fatty acid binding protein	Aca s 13		Lep d 13	Tyr p 13		Blo t 13	Der f 13	
14	Apolipophorin							Der f 14	Der p 14
15	Chitinase							Der f 15	
16	Gelsolin/villin							Der f 16	
17	Ca binding protein							Der f 17	

18	Chitinase							Der f 18	
19	Anti-microbial peptide						Blo t 19		
20	Arginine kinase								Der p 20
21	???						Blo t 21		Der p 21
22	???							Der f 22	
23	???								Der p 23
24	Troponin C				Tyr p 24				

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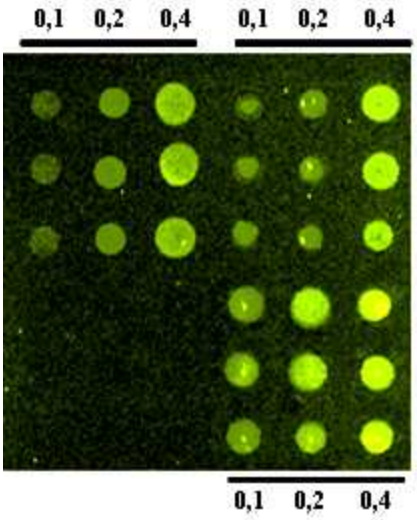

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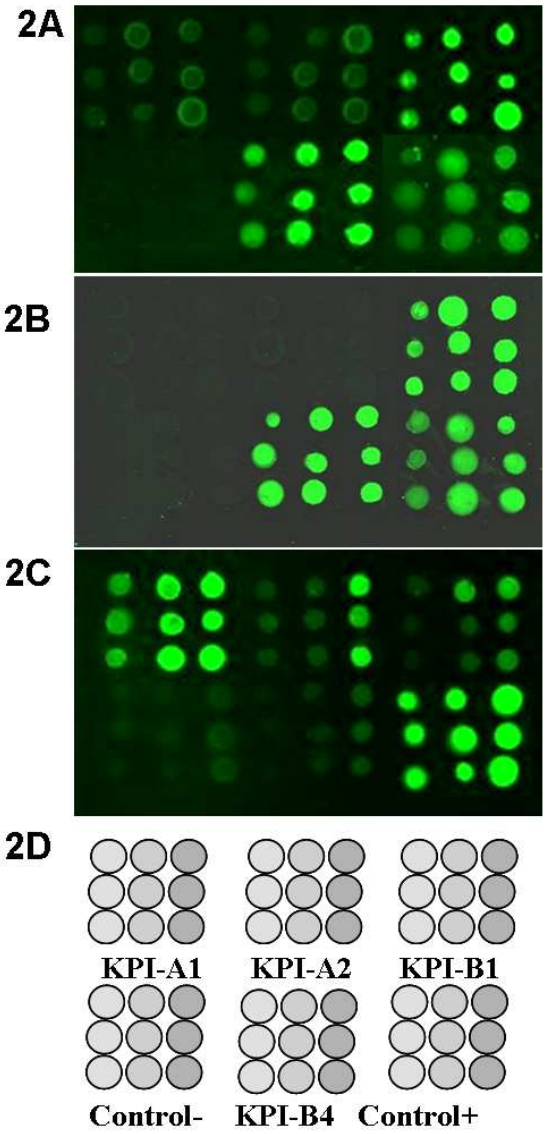

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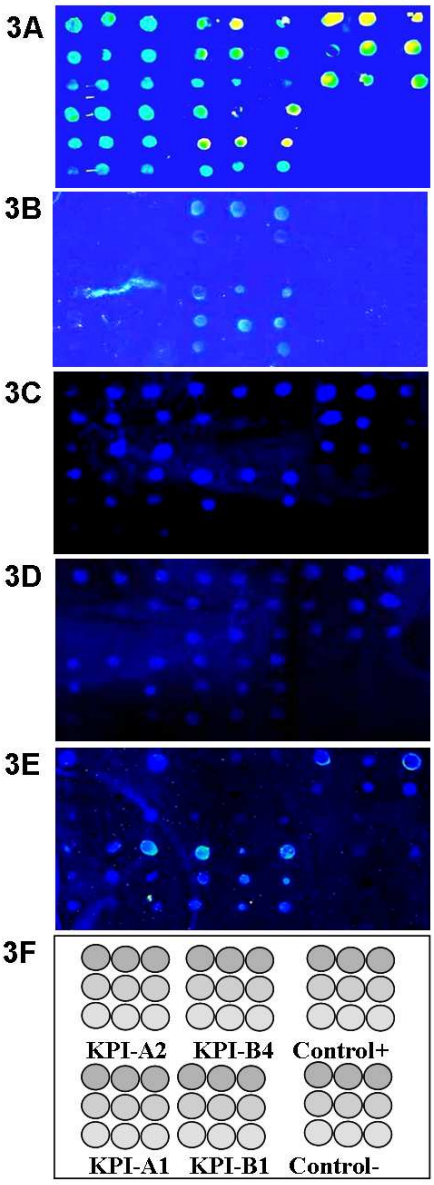

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