

Short communication

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

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Stored-food and house-dust arthropods include many species of mites and beetles that affect 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.

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

We previously 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 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 limited only 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, it is highly feasible that KPI groups contain protease inhibitors could inhibit trypsin from different origins.

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

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 mycotoxin-producing fungi

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Abbreviations: KPI, Kunitz-type protease inhibitor; S-BBI, soybean Bowman-Birk protease inhibitor

[9]. More than 20 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' feces.

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 for detecting mite proteins supposed to be proteases. Protein visualization of anti-mite antibodies allows relative quantification of mite proteases bound to each KPI group in each individual experiment, but not 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.

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-Aldrich (Milwaukee, WI, USA).

All recombinant protease inhibitors were desalted with Zeba desalt spin columns (Pierce, Rockford, IL, USA). The KPIs and the positive control, soybean Bowman-Birk inhibitor (S-BBI), were prepared at different concentrations (0.1, 0.2, and 0.4 mg/mL) in NaHCO_3 buffer (0.1 M, pH 9) with 40% glycerol and arrayed on epoxysilane-coated Nexterion slides (Schott, 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 0.1, 0.2 or 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 2 h at 60% humidity in a dark room to allow the reaction between epoxy groups and water. The printed slides were stored dry in the

dark for later use, or immediately used. The protein chips ready for use were incubated for 1 h on a shaker at room temperature in PBS containing 0.5 M glycine and 1% BSA to block nonspecific binding. Subsequently, the slides were washed with PBS + 0.1% BSA and dried in a centrifuge at $500 \times g$ for 2 min.

Each protease (trypsin, chymotrypsin and cathepsin B) was labeled with an Alexa-555 protein labeling 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 labeled, purified protease (1 mg/mL trypsin and chymotrypsin in 0.1 M TRIS buffer, pH 7.5; and 1 mg/mL cathepsin B dissolved in 0.1 M phosphate buffer, pH 6.5) was applied with the hybridization buffer onto the slide, using the coverslip method [12–14], and incubated for 1 h in the dark at room temperature in a humidity chamber (70% humidity). After incubation, the slides were washed with PBS containing 0.2% Tween-20 and 0.1% BSA for 15 min on a shaker at 4°C. The slides were then washed twice with PBS + 0.05% BSA for 5 min on a shaker at 4°C and dried in a centrifuge at $500 \times g$ for 2 min. The slide was scanned with a Laser Scanner 428 (Affymetrix, CA, USA).

For investigating mite extracts, protease inhibitors (KPI-A1, -A2, -B1, -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 (~500 nL). After protein immobilization and slide desiccation, the chips were used the following day.

Mite cultures (*Acarus siro*, *Aleuroglyphus ovatus*, *Lepidoglyphus destructor*, *Tyrophagus putrescentiae*), 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 mites were obtained from starved, washed mites. Frozen adults were weighed and homogenized in PBS (20 mM Na_2HPO_4 , 20 mM NaH_2PO_4 , 150 mM NaCl, pH 7) and 0.1% Triton X-100. Protein concentration was determined using the Bradford method (Bio-Rad), and were: *A. ovatus* extract, 3.5 $\mu\text{g}/\mu\text{L}$; *A. siro* extract, 2.9 $\mu\text{g}/\mu\text{L}$; *L. destructor* extract, 3.2 $\mu\text{g}/\mu\text{L}$; *T. putrescentiae* extract, 2.8 $\mu\text{g}/\mu\text{L}$; and *T. castaneum* extract, 2 $\mu\text{g}/\mu\text{L}$. The protein extract (2–2.5 $\mu\text{g}/\mu\text{L}$) was diluted with PBS; 20 μL hybridization solution containing 1 μL of 1:10 dilution was used, so that final concentration of proteins was between 0.2 and 0.3 $\mu\text{g}/\mu\text{L}$. The slides were hybridized in the dark at room temperature on a microplate shaker, and

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

Class	Biochemical function	Stored-product species			Dust species			
		<i>Acarus siro</i>	<i>Glyciphagus domesticus</i>	<i>Lepidoglyphus destructor</i>	<i>Tyrophagus putrescentiae</i>	<i>Aleuroglyphus ovatus</i>	<i>Blomia tropicalis</i>	<i>Dermatophagoides farinae</i> <i>Dermatophagoides pteronyssinus</i>
1	Cysteine protease				a)	a)	Blot 1	Der p 1
2	NPC2 family		Gly d 2	Lep d 2	Tyr p 2		Blot 2	Der p 2
3	Trypsin	a)		a)	a)	a)	Blot 3	Der p 3
4	A-amylase	b)		b)	b)	b)	Blot 4	Der p 4
5	■ ^c			Lep d 5			Blot 5	Der p 5
6	Chymotrypsin	b)		b)	b)	b)	Blot 6	Der p 6
7	■ ^c			Lep d 7			Der f 6	Der p 7
8	Glutathione S-transferase						Der f 7	Der p 8
9	Collagenolytic serine prot.	b)		b)	b)	b)		Der p 9
10	Tropomyosin			Lep d 10	Tyr p 10		Blot 10	Der p 10
11	Paramyosin						Blot 11	Der p 11
12	???						Blot 12	
13	Fatty acid binding protein	Aca s 13		Lep d 13	Tyr p 13		Blot 13	
14	Apolipophorin						Der f 13	Der p 14
15	Chitinase						Der f 14	
16	Gelsolin/villin						Der f 15	
17	Ca binding protein						Der f 16	
18	Chitinase						Der f 17	
19	Anti-microbial peptide						Der f 18	
20	Arginine kinase						Blot 19	Der p 20
21	■ ^c							Der p 21
22	■ ^c						Blot 21	
23	■ ^c						Der f 22	Der p 23
24	Troponin C				Tyr p 24			

a) Suggested allergens according to their amino acid sequences and homology to known allergens.

b) Enzymatic activity proved.

c) ■, unknown function.

were 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-labeled protein A for the antibody detection. Slides were analyzed with a 428 Laser Scanner, and images were visualized with ScanAnalyse. The sensitivity of antigen detection on protein chips was compared to results obtained using dot blot and sandwich-ELISA assays probed with the same polyclonal antibodies [15–17].

Protein chips were tested comparing trypsin binding to S-BBI, KPI-B1 and KPI-B4 recombinant proteins [4] to verify their performance under conditions of limited movement and orientation constraint (Fig. 1), Miniaturization of the protein chip was achieved using a robotic spot-arrayer, depositing 70-nL volumes onto the glass. KPI-B1 and -B4 at serial dilutions showed affinity for trypsin (10 ng/µL) even at 0.1 ng/nL (Fig. 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 using KPI-B1 in biotechnological applications.

We also investigated the stability of KPI protein chips. A set of printed slides was stored for 1 month at 4°C and their performance was tested. No evident differences were detected compared to a freshly printed chip. KPIs were stable for a long time without loss of activity.

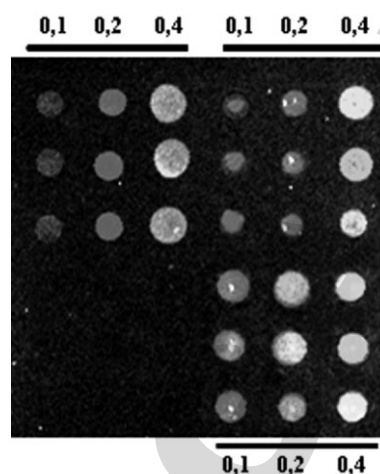


Figure 1. Miniaturized protein chips in detection of fluorescent trypsin. Upper lane: KPI-B1 (left), KPI-B4 (right). Lower lane: BSA, S-BBI.

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

The application of KPIs to detect mite [15, 16] or insect [17] proteases as potential allergens [18, 19] was examined. 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 (Figs. 3A–E). Opti-

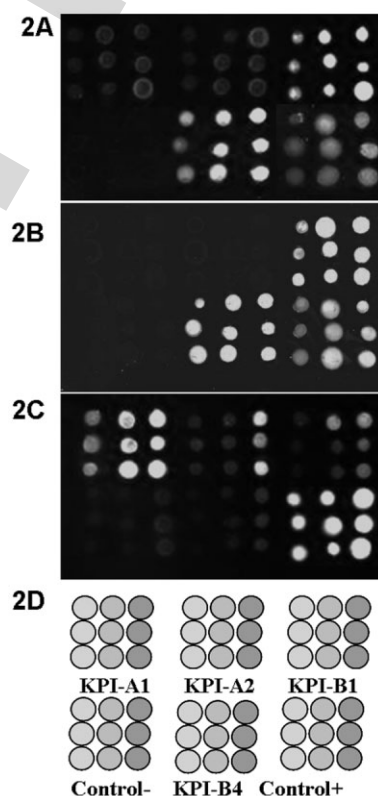


Figure 2. Detection of three fluorescent proteases on KPI chips. Protein concentrations: 0.1, 0.2 and 0.4 ng/nL (left to right). (A) Selective binding of trypsin. (B) Selective binding of chymotrypsin. (C) Selective binding of cathepsin B. (D) A scheme of spotted proteins. Upper lane: KPI-A1, KPI-A2 and KPI-B1. Lower lane: negative control (BSA), KPI-B4, positive control [S-BBI in (A, B); lactoferrin in (C)].

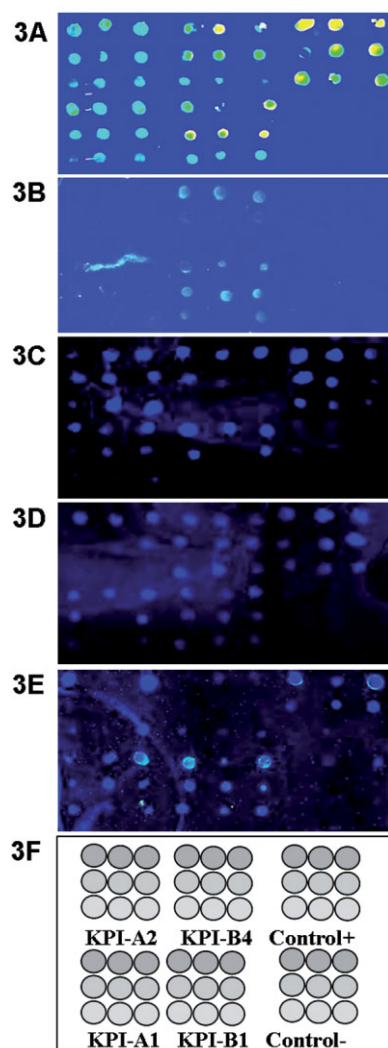


Figure 3. Detection of mite proteases using KPI chips. Detection of (A) *Aleuroglyphus ovatus* using anti-*A. ovatus* antibodies; (B) *Acarus siro* using anti-*A. siro* antibodies; (C) *Lepidoglyphus destructor* using anti-*L. destructor* antibodies; (D) *Tyrophagus putrescentiae* using anti-*T. putrescentiae* antibodies; and (E) *Tribolium castaneum* using anti-*T. castaneum* antibodies. (F) Scheme of protein spots (upper lane 0.4 ng/nL; middle lane 0.2 ng/nL; lower lane 0.1 ng/nL).

imum resolution shown in Fig. 3 was achieved with 1:10 extract dilution, corresponding to 0.2 µg total proteins, but positive results were obtained even with extract dilutions as low as 1:20 (corresponding to 100 ng 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, polysaccha-

rides and chitins that may block the protein interactions on the chip.

For 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 total mite extract, while the detection limit for sandwich-ELISA method is 1 µg mite proteins using 1 mL solutions. An advantage of the proposed method is the limited hybridization volume with lower amounts of proteins, due to the very small surface of protein chips. However, considering that proteases represent only a small fraction of body proteins, assuming this to be less 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.

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. At present, 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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The authors have declared no conflict of interest.

References

- [1] Valueva, T. A., Mosolov, V. V., Role of inhibitors of proteolytic enzymes in plant defence against phytopathogenic microorganisms. *Biochemistry (Mosc.)* 2004, 69, 1305–1309.
- [2] Heibges, A., Salamini, F., Gebhardt, C., Functional comparison of homologous members of three groups of Kunitz-type enzyme inhibitors from potato tubers (*Solanum tuberosum* L.). *Mol. Gen. Genomics* 2003, 269, 1215–1221.
- [3] Speransky, A. S., Cimaglia, F., Krinitsina, A. A., Poltronieri, P. et al., Kunitz-type protease inhibitors group B from *Solanum palustre*. *Biotechnol. J.* 2007, 2, 1417–1424.
- [4] Rawlings, N. D., Tolle, D. P., Barrett, A. J. Evolutionary families of peptidase inhibitors. *Biochem. J.* 2004, 378, 705–716.
- [5] Stejskal, V., Hubert, J., Risk of occupational allergy to stored grain arthropods and false pest-risk perception in Czech grain stores. *Ann. Agric. Environ. Med.* 2008, 15, 29–35.

- [6] Stewart, G. A., Lake, F. R., Thompson, P. J., Faecally derived hydrolytic enzymes from *Dermatophagoides pteronyssinus*: Physicochemical characterization of potential allergens. *Int. Arch. Allergy Appl. Immunol.* 1991, **95**, 248–256.
- [7] Shakib, F., Gough, L., The proteolytic activity of Der p 1 selectively enhances IgE synthesis: A link between allergenicity and cysteine protease activity. *Clin. Exp. Allergy* 2000, **30**, 751–752.
- [8] Stewart, G. A., Robinson, C., The immunobiology of allergenic peptidases. *Clin. Exp. Allergy* 2003, **33**, 3–6.
- [9] Hubert, J., Stejskal, V., Kubátová, A., Munzbergová, Z. *et al.*, Mites as selective fungal carriers in stored grain habitats. *Exp. Appl. Acarol.* 2003, **29**, 69–87.
- [10] van Amerongen, A., Ostrafe, V., Gruppen, H., Meerdink, G. *et al.*, Specific immuno-(chymo)trypsin inhibitor assays for determination of (residual) activity of Bowman-Birk or Kunitz soybean trypsin inhibitors. In: Jansman, G. D., Hill, J., Huisman, J., van der Poel, A. F. B. (Eds.), *Recent advances of research in antinutritional factors in legume seeds and rapeseed*. Wageningen Pers, Wageningen, 1998, pp. 32–37.
- [11] van Amerongen, A., Wichers, J. H., Berendsen, L. B., Timmermans, A. J. *et al.*, Colloidal carbon particles as a new label for rapid immunochemical test methods: Quantitative computer image analysis of results. *J. Biotechnol.* 1993, **30**, 185–195.
- [12] Haab, B. B., Dunham, M. J., Brown, P. O., Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. *Genome Biol.* 2001, **2**, Research0004.1–0004.13.
- [13] Templin, M. F., Stoll, D., Schwenk, J. M., Pötz, O. *et al.*, Protein microarrays: Promising tools for proteomic research. *Proteomics* 2003, **3**, 2155–2166.
- [14] Cretich, M., Damin, F., Pirri, G., Chiari, M., Protein and peptide arrays: Recent trends and new directions. *Biomol. Eng.* 2006, **23**, 77–88.
- [15] Krizkova-Kudlikova, I., Stejskal, V., Hubert J., Comparison of detection methods for *Acarus siro* (Acari: Acaridida: Acarididae) contamination in grain. *J. Econ. Entomol.* 2007, **100**, 1928–1937.
- [16] Kudlikova, I., Stejskal, V., Hyblova, J., Chalupnikova, J. *et al.*, Polyclonal antibodies for detection of flour mite *Acarus siro* (Acari: Acaridida). *Phytophaga* 2004, **14**, 711–720.
- [17] Krizkova-Kudlikova, I., Hubert, J., Development of polyclonal antibodies for the detection of *Tribolium castaneum* contamination in wheat grain. *J. Agric. Food Chem.* 2008, **56**, 8035–8040.
- [18] Erban, T., Hubert, J., Comparative analyses of proteases in seven species of synanthropic acaridid mites. *Arch. Insect Biochem. Physiol.* 2010 (in press).
- [19] Erban, T., Erbanova, M., Nesvorna, M., Hubert, J., The importance of starch and sucrose digestion in nutritive biology of synanthropic acaridid mites: Amylases and glucosidases are suitable targets for inhibitor-based strategies of mite control. *Arch. Insect Biochem. Physiol.* 2009, **71**, 139–158.
- [20] Tong, J. C., Lim, S. J., Muh, H. C. *et al.*, Allergen atlas: A comprehensive knowledge center and analysis resource for allergen information. *Bioinformatics* 2009, **25**, 979–980.
- [21] Mari, A., Rasi, C., Palazzo, P., Scala, E., Allergen databases: Current status and perspectives. *Curr. Allergy Asthma Rep.* 2009, **9**, 376–383.

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