



PROTEIN CHIPS FOR ANALYSIS OF KPIs IN POTATO RESPONSE TO FUNGI AND IN DETECTION OF PROTEASE ALLERGENS

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Poltronieri P

COST 853 "Agricultural biomarkers for array technology"

"ARRAY" **A**dvanced **R**esearch **R**esults using **A**rray technology
strategic Workshop, COST Food and Agriculture Domain committee
S. Feliu de Guixols, 22-24.5.2007

Molecular analyses on macroarrays and microplate formats

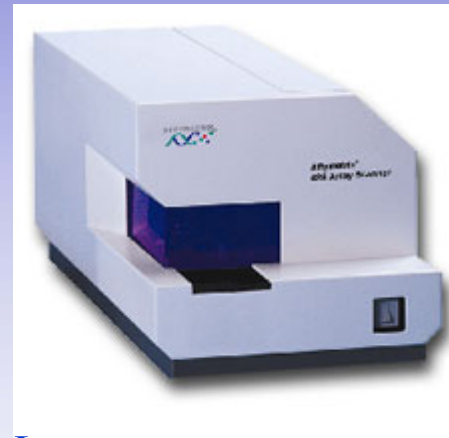
Array technology allows high-throughput analysis of a series of compounds



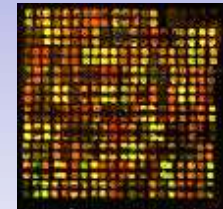
Hybridisation with a labeled probe



SpotArray 24 Perkin Elmer

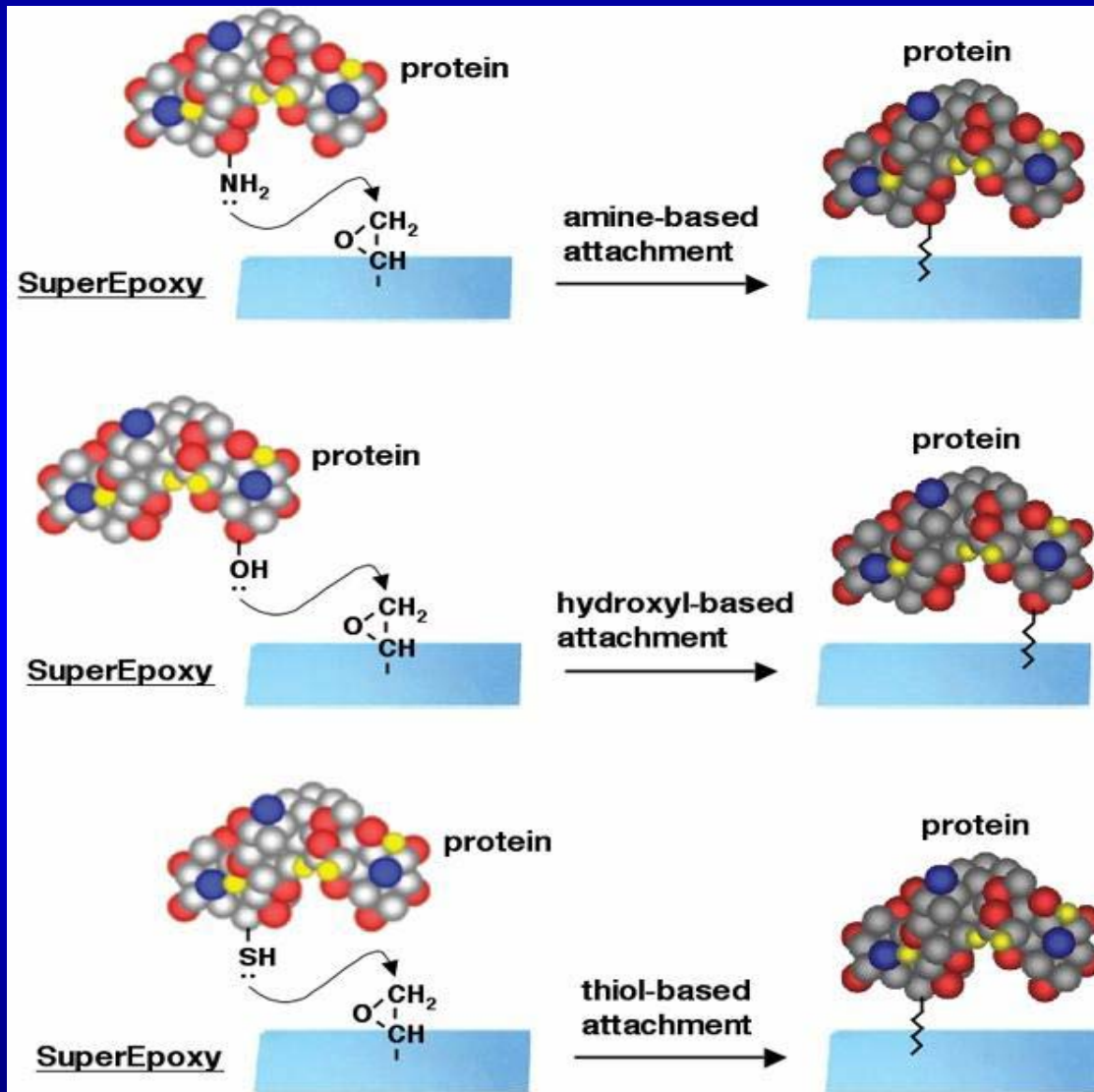


Laser scanner



Signal Analysis of fluorescence intensity on a palette color scale

Types of binding of proteins on Epoxy-slides



ANALYSIS OF KUNITZ-TYPE PROTEASE INHIBITORS

Potato family of Kunitz-type Proteinase inhibitors (KPIs) are 22 kDa proteins expressed constitutively or induced tissue-specifically by fungi, with a defensive function.

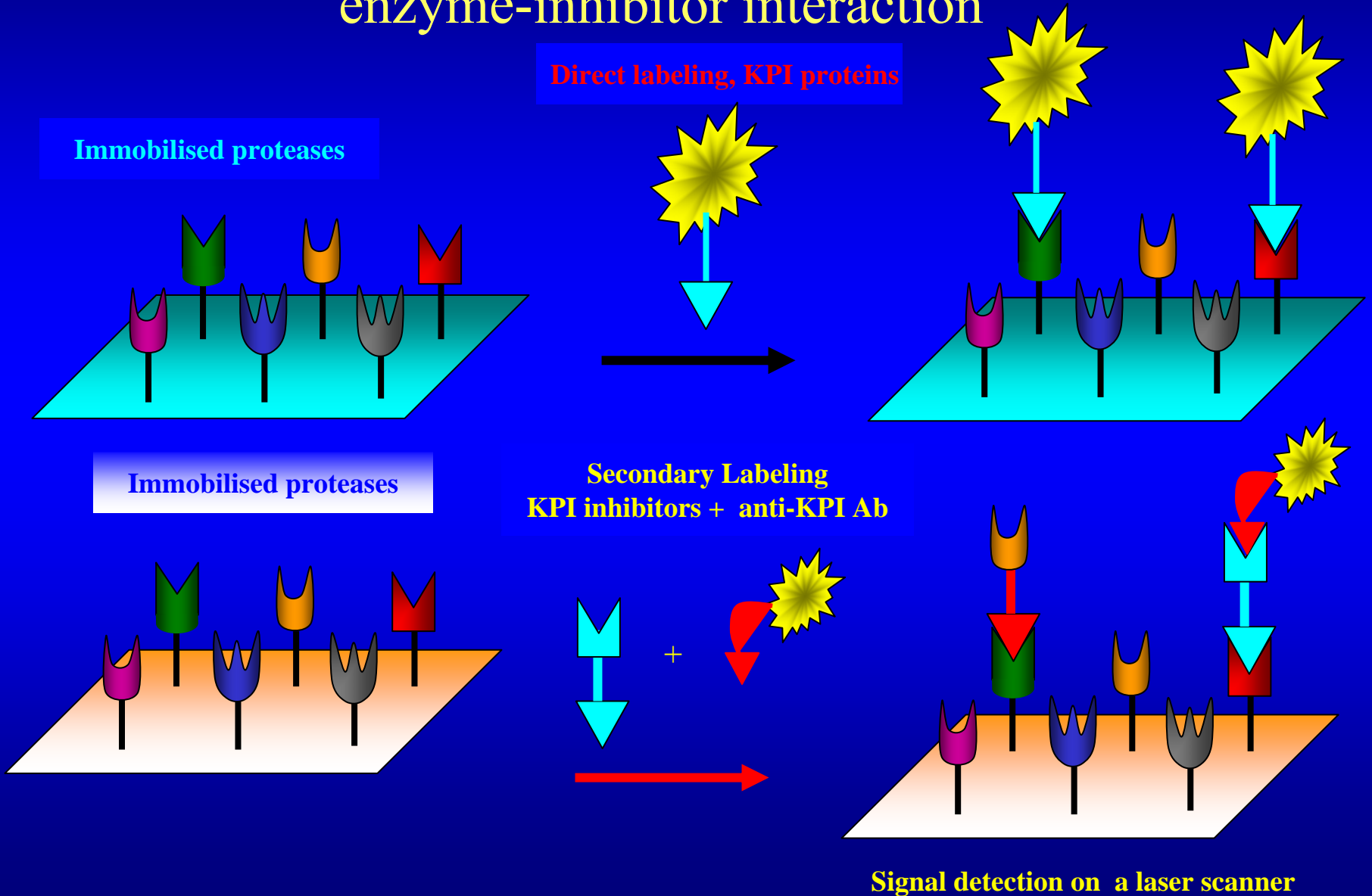
- Solanaceae genomes (*S. tuberosum*, *S. stoloniferum*, *S. palustre*) contain various Kunitz-type proteinase inhibitors, of group A (blocking aspartic and serine proteases), group B (blocking serine proteases) and group C (blocking cysteine proteases and other hydrolases)
- Wild and tuber-forming potato varieties (Provita, Saturna, Istrinkii, Bintjie, Elkana) express many KPI isoforms (up to 21-30 genes), producing a wide array of inhibitors against pest proteases and hydrolases.

Protease- chips

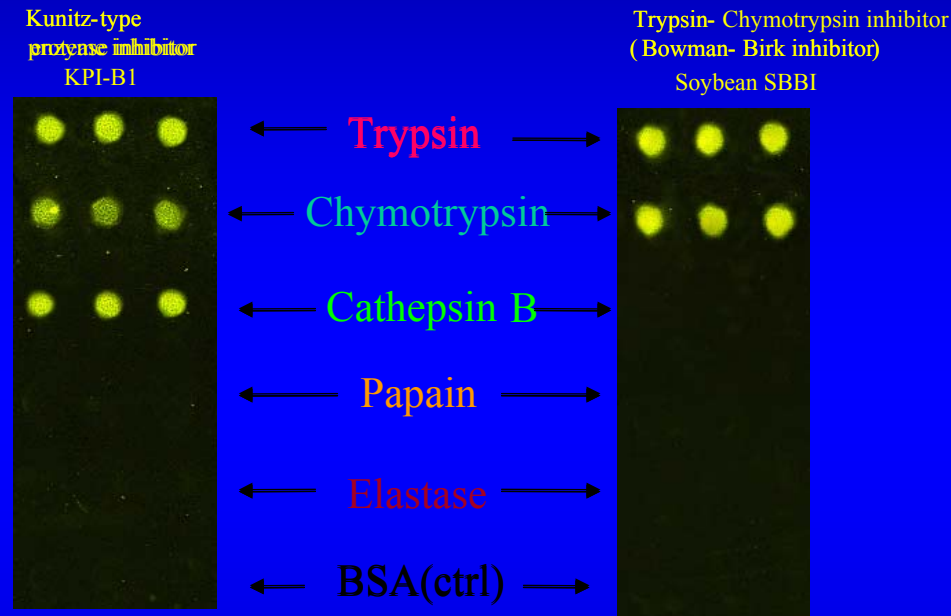
in detection of differential expression of Kunitz-type protease inhibitors during fungal infection

- Protease chips were used to quantify the amount of fungi-induced Kunitz-type protease inhibitors
- In parallel, we quantified the anti-protease activity by in vitro inhibition assays
- We used recombinantly-expressed potato KPI proteins to quantify KPIs by anti-KPI antibodies, and fluorescence detection using direct and indirect detection methods

Principles of protein chip technology in the detection of enzyme-inhibitor interaction



Protease chips: hybridization with labeled KPI-B1 Comparison with soybean BBI



proteases	KPIB1
trypsin	+
chymotrypsin	+
Cathepsin B	+
papain	-
elastase	-

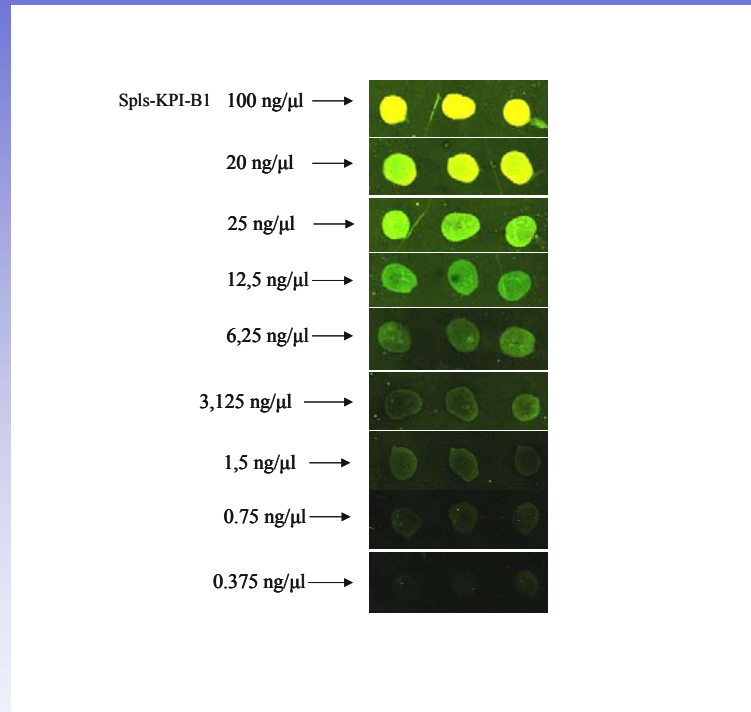
In vitro inhibition activity

Proteases were spotted in triplicate, 0,5 $\mu\text{g}/\mu\text{l}$ in glycerol 40%. Hybridization was performed with kunitz-type protease inhibitor B1 (recombinant protein) and S-BBI, the soybean Bowman-Birk trypsin-chymotrypsin inhibitor as control, both labelled with NHS-Alexa-555.

In parallel, biochemical characterisation of protease inhibition was assessed.

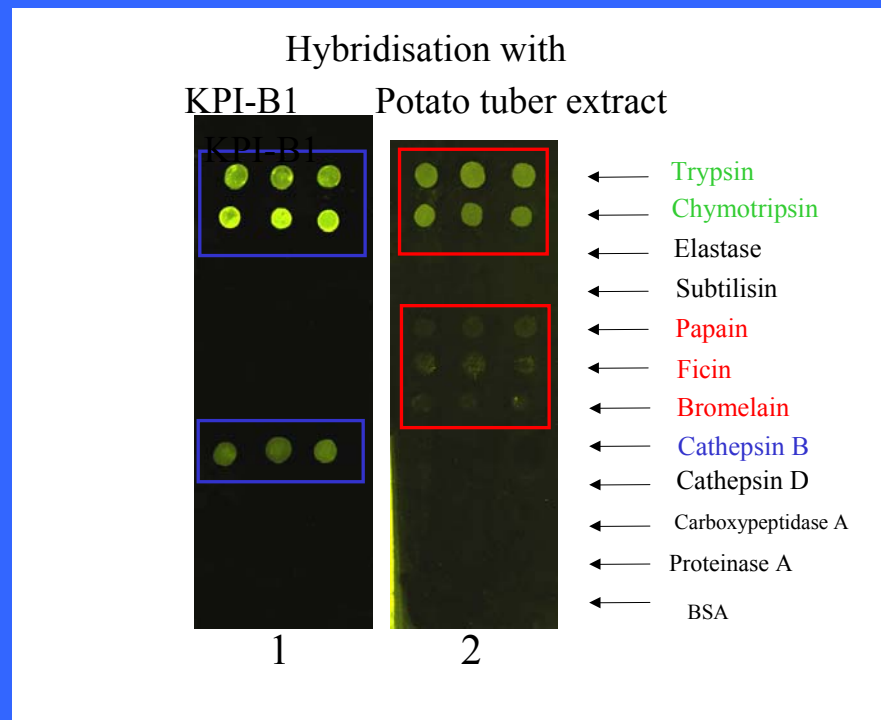
Protease chips

hybridization at various dilutions of protease inhibitor KPI-B1



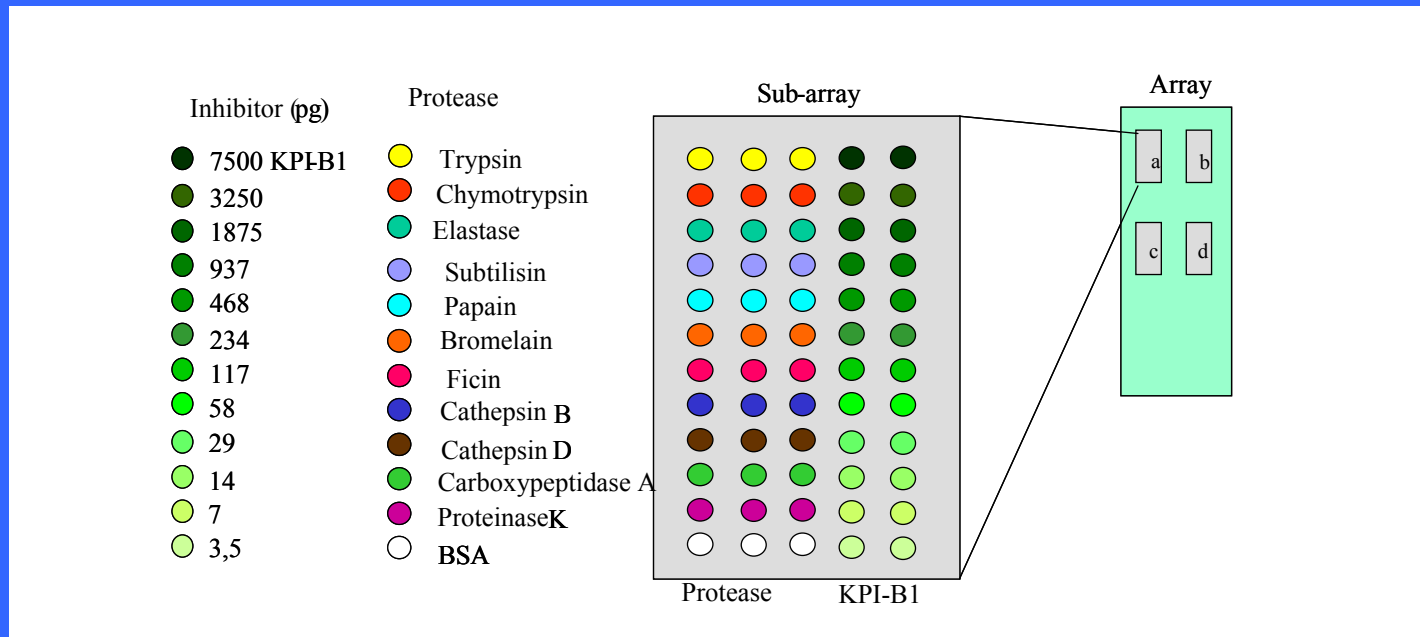
Trypsin was spotted in triplicate on the chip surface at $1\mu\text{g}/\mu\text{l}$ concentration. Hybridisation at decreasing concentrations of KPI-B1, labelled with Alexa-555, Considering $10\mu\text{l}$ volumes of hybridisation solution used, the sensitivity limit of the assay is approximately 10 ng of total KPI protein ($1\text{ ng}/\mu\text{l}$)

Protease chips for quantitative analysis of KPI binding



Protease-chips, one incubated with KPI-B1 (1) and the second with potato tuber extract (2). Detection with anti-KPI antibody labelled with Alexa-555.

KPI-B1 Reference control to quantify fluorescence intensity due to anti-KPI Abs binding

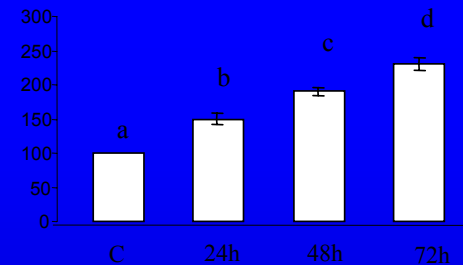
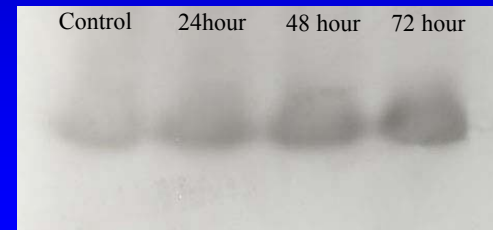


Each Sub-array was incubated with potato tuber extracts: a) uninfected extract (control); b) extract infected at 24 h; c) extract infected at 48 h; d) extract infected at 72 h.

Anti-KPI Alexa-555-labeled antibody-based recognition of KPIs from tuber extracts bound to the proteases on the chip; signals from reference KPI-B1 spots used to compare the intensity of Ab binding, as quantitative evaluation.

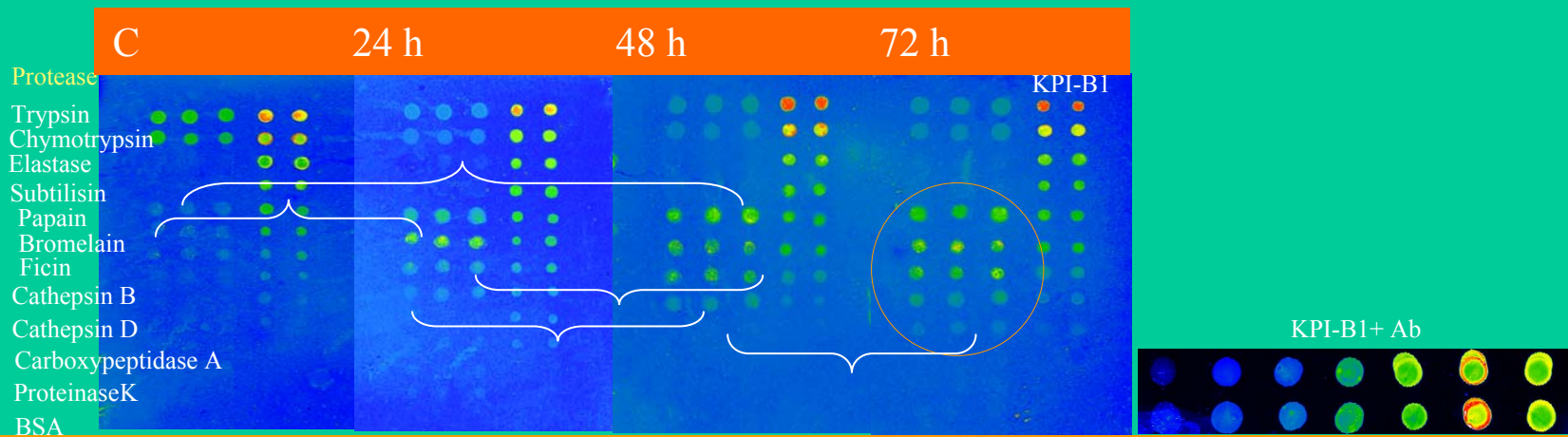
Detection of biomarkers of fungal infection

Western blot of constitutive KPIs and KPI induced by fungal infection in potato tubers



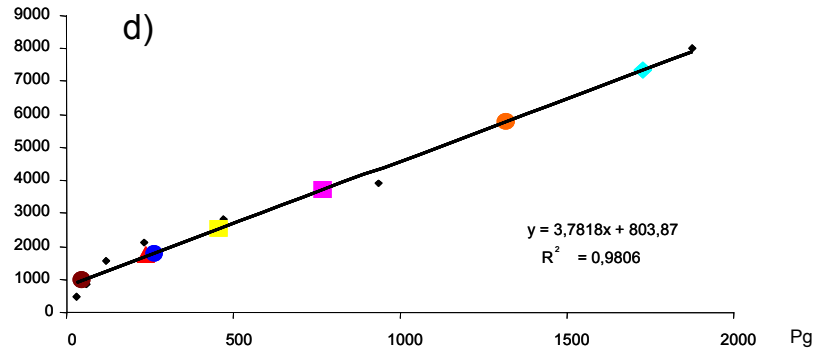
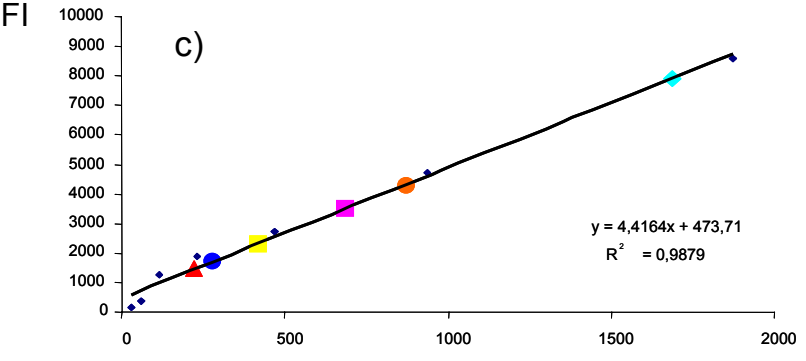
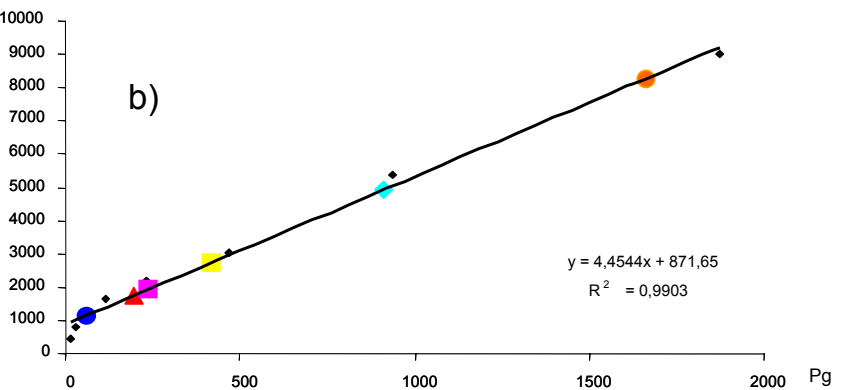
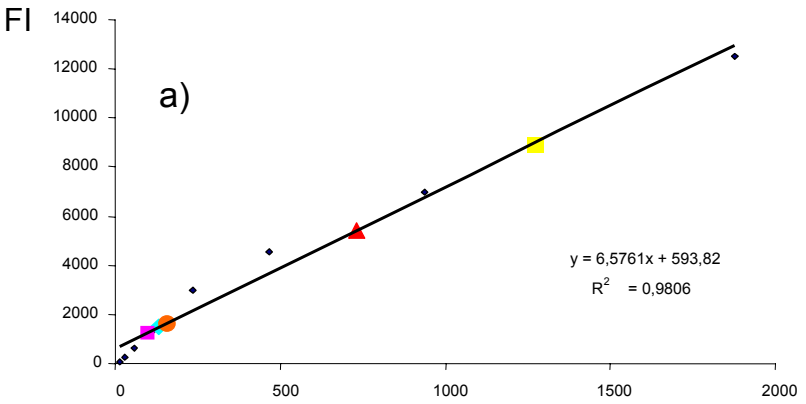
Potato tuber slices were grown in Petri dishes inoculated with fungal spores. Immunoblots made using anti-KPI antibodies /secondary Ab chemiluminescence. Densitometric analysis (number of pixels per area unit) of bands relative to KPI-B +KPI-A protease inhibitors in potato tuber extracts (*S. tuberosum*) infected with *Aspergillus carbonarius* after 24, 48 and 72 hours. Results expressed in percentage in respect to control considered 100% of value. Values obtained are significantly different, according to Fisher test PLSD (ANOVA $p < 0.0001$). Five independent experiments analysed.

Quantification of KPIs induced by fungal infection through relative signal intensity using KPI-B1 as reference



Time course experiments of fungal infection, hybridisation of tuber extracts on the protease arrays, detection with anti-KPI Antibodies, and calibration with signals from KPI-B1 serial dilutions used as Ab-binding control. a) uninfected potato, control extract; b) potato extract 24h infection; c) potato extract 48 h infection; d) potato extract 72 h infection.

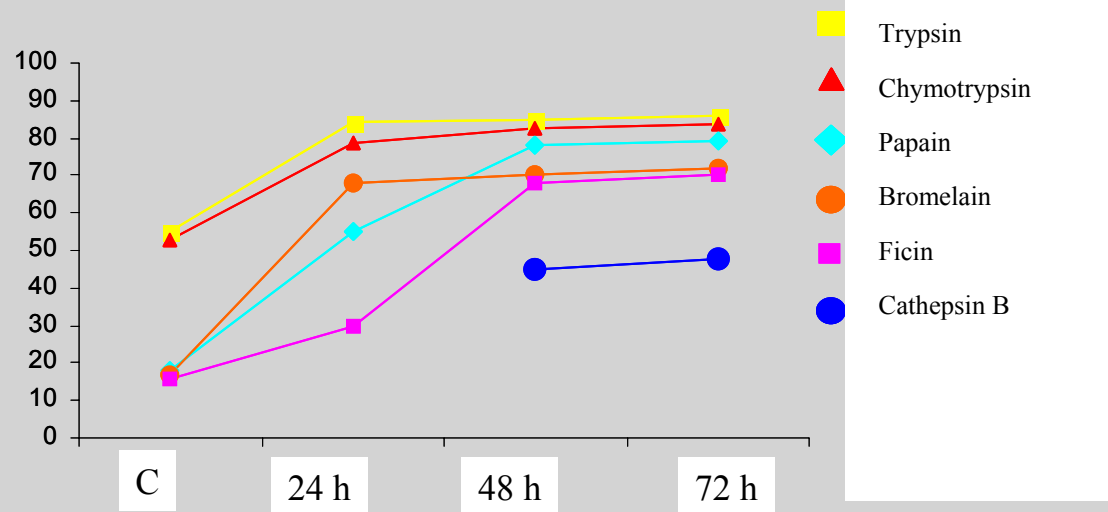
Plots of signal intensity data of KPIs binding to each protease



- KPI anti-Trypsin
- ▲ KPI anti - Chymotrypsin
- ◆ KPI anti-papain
- KPI anti - -Bromelain
- KPI anti- -Ficin
- KPI anti - cathepsin B
- KPI anti - cathepsin D

Fluorescence intensity (FI) of signals, in picograms of KPIs in tuber extracts: a) uninfected control; b) infected at 24 h; c) infected at 48 h; d) infected at 72 h. ScanAnalyze Evaluation calibration curve, as fluorescence intensity in function of Pg: picograms of immobilized KPI-B1 protein stained by anti-KPI Ab. Papain-bound KPIs increased totally from 133 picograms in control (a) to 1500 pg at 48 /72 hr

Screening of potato varieties for anti-protease activity: differential KPI expression using biochemical assays



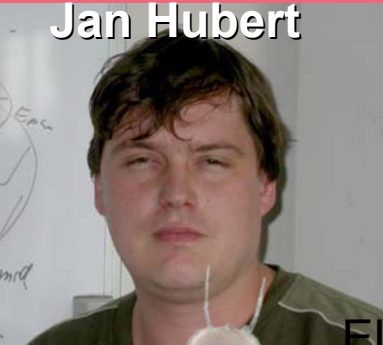
Biochemical assays of protease inhibition by KPIs expressed in tuber extracts

Trypsin, chymotrypsin, bromelain, papain, ficin and cathepsin B were found inhibited at various stages of infection. (C: ctrl; *A. carbonarius* infected tubers: 24, 48, 72 hr).

Detection of KPIs with protease-chips correlated with anti-protease activity in vitro assays, except for anti-trypsin activity, increasing in biochemical assays, but not in protein chips: absence of detection of anti-trypsin KPI was due to competitive interaction with newly expressed low molecular weight PI-I and PI-II.

ANTI-MITE ANTIBODY CHIPS AND SANDWICH METHODS FOR DETECTING MITE ALLERGENS

Jan Hubert



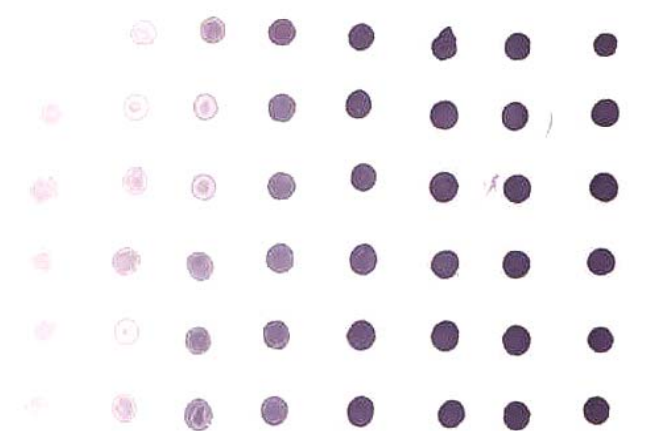
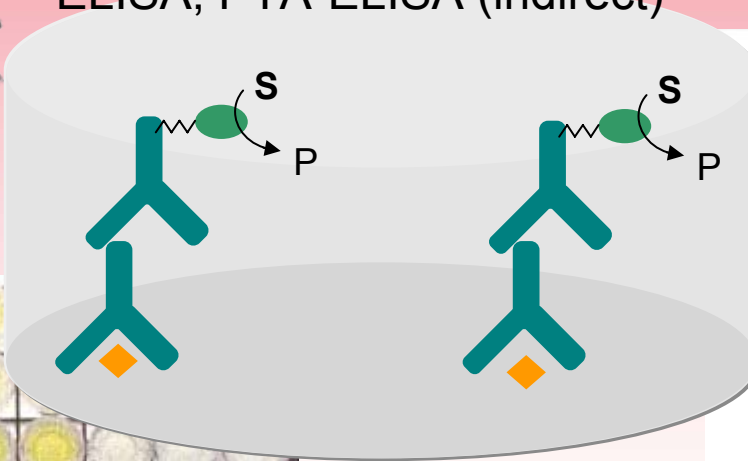
Iva Kudlíková



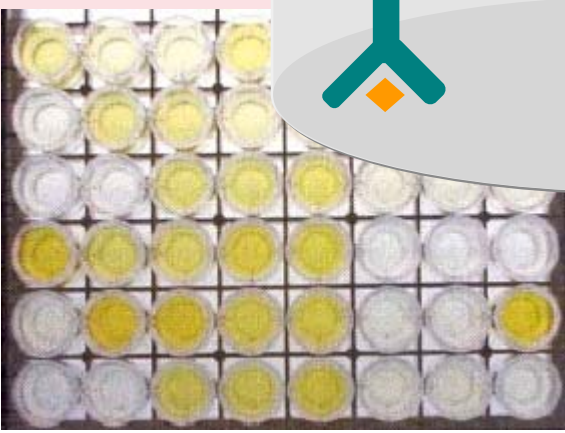
Comparative analysis

ELISA, PTA-ELISA (indirect)

Dot-Blot

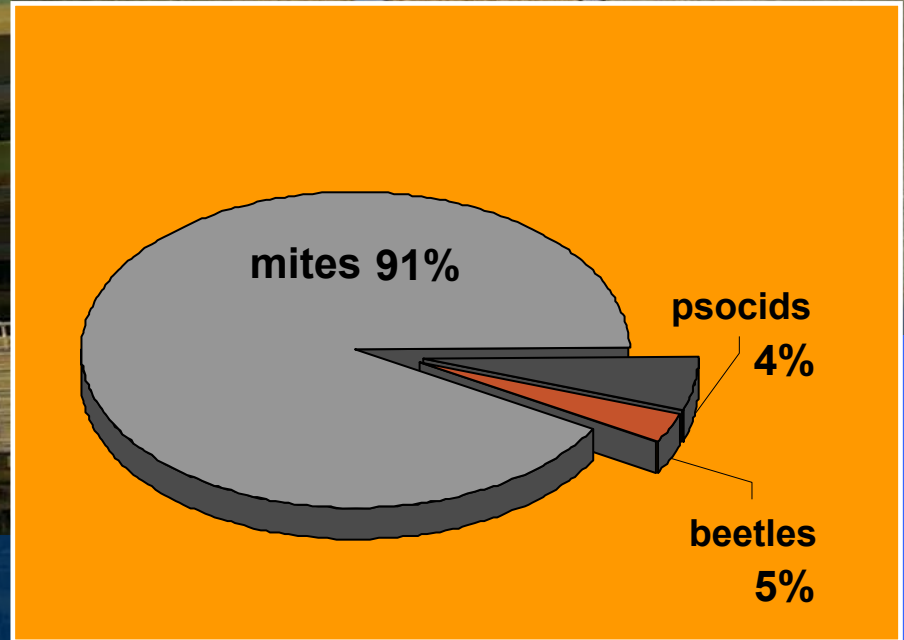
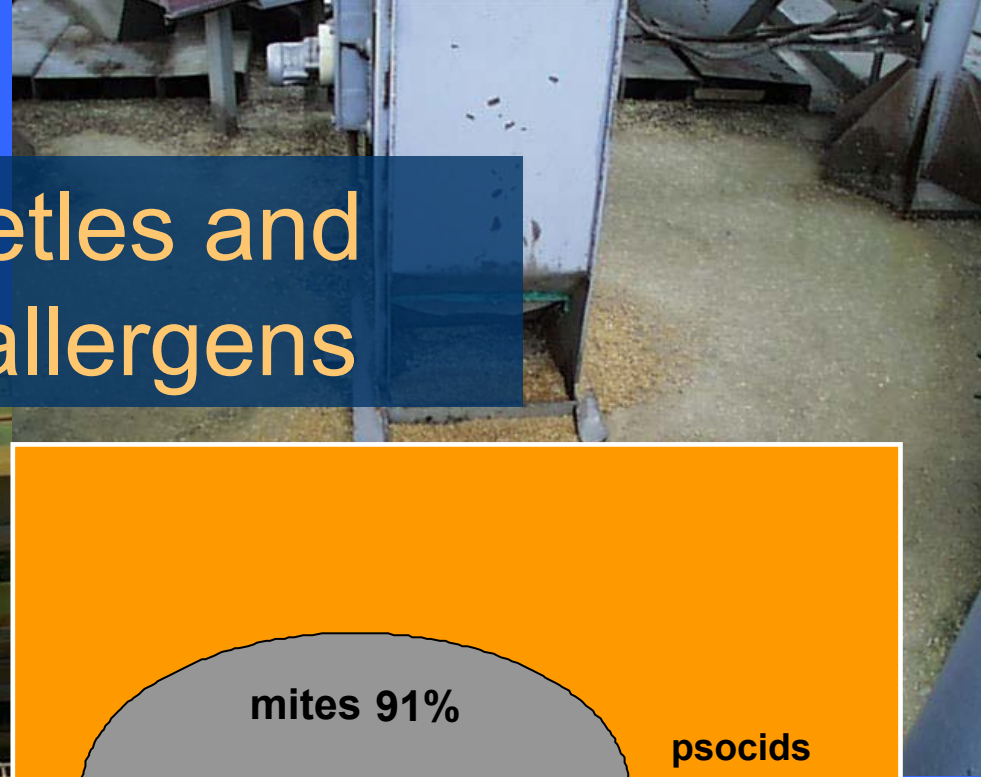


0,1	0,5	1	2,5	5	10	25	50	75	100
µg/ml		Concentration							



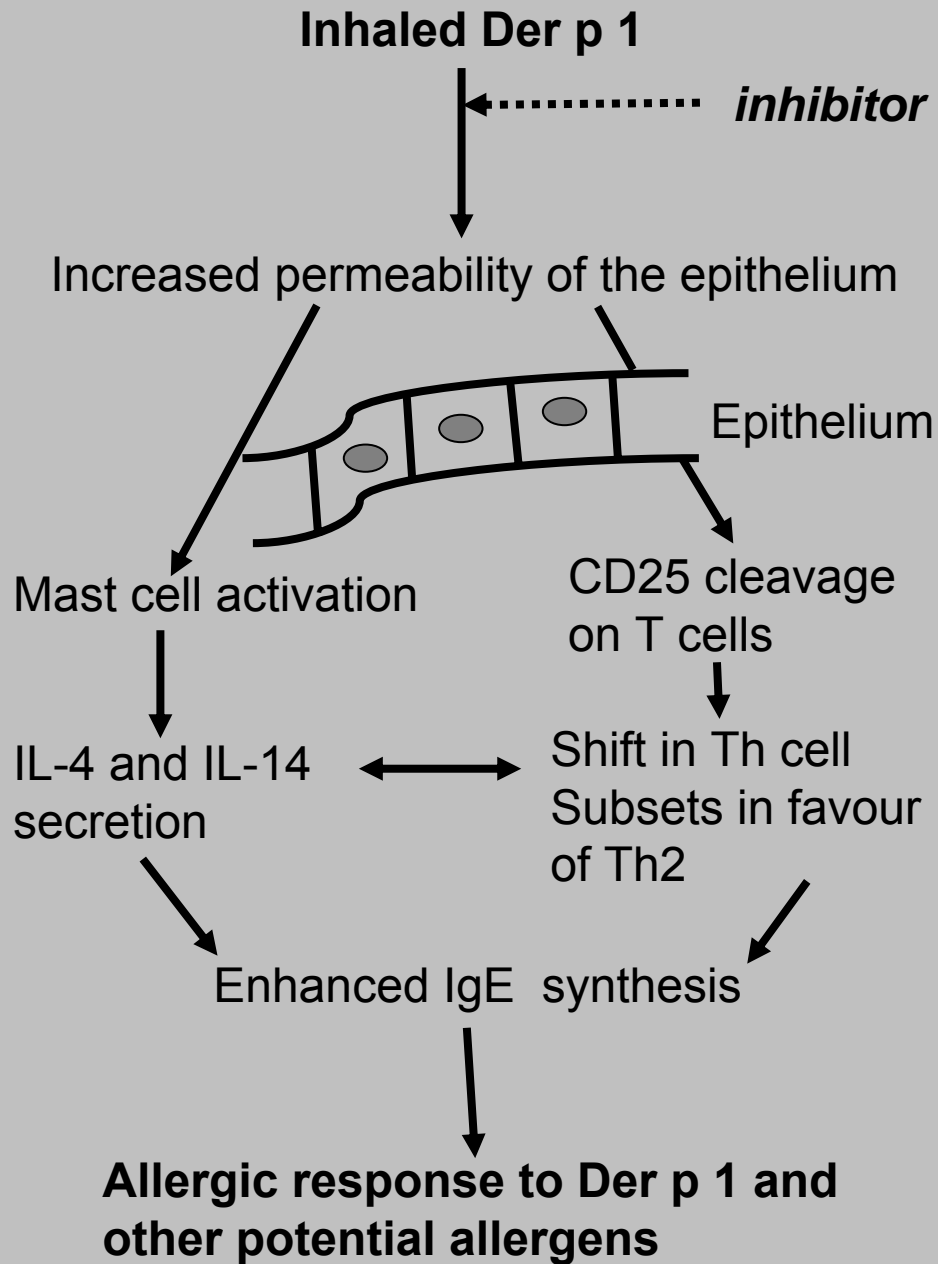
Mite cultures and immunization done at VURV in Prague

Stored product beetles and mites – source of allergens



To develop friendly detection and monitoring system of pest contamination for farmers and state officers

Class	Biochemical function	Stored product species				Dust species		
		<i>Ac. siro</i>	<i>Gly. domesticus</i>	<i>Lep. destructor</i>	<i>Tyr. putrescentiae</i>	<i>Blo. tropicalis</i>	<i>Dermatoph. farinae</i>	<i>Dermatoph. pteronyssinus</i>
1	Cysteine prot	Aca s 1	Gly d 1	Lep d 1	Tyr p 1	Blo t 1	Der f 1	Der p 1
2	Aspartic protease							
3	Trypsin	Aca s 3		Lep d 3	Tyr p 3	Blo t 3	Der f 3	Der p 3
4	Amylase					Blo t 4		Der p 4
5								
6	Chymotrypsin			Lep d 6		Blo t 6	Der f 6	Der p 6
7								
8	Glutathionate-S-transferase							
9	Collagenolytic serine protease			Lep d 9				Der p 9
10	Tropomyosin							
11	Paramyosin							
12								
13	Fatty acid binding prot							
14	Vitellogenin/Apolipoporphin							
15	Chitinase			Lep d 15				
16	Gelsolin/villin							
17	Ca binding protein							
18	Lysozyme-like							
19	Anti-microbial peptide							
20	Arginine kinase							



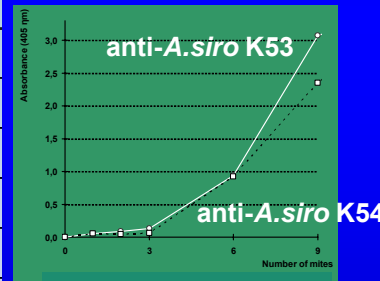
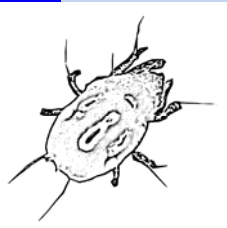
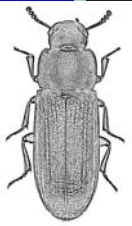
Antibodies raised against protein fraction from *T. putrescentiae* whole body homogenates

ORDER	SUBORDER	FAMILY	SPECIES	Relative assay response (%)		
				concentration 1µg	10µg	
Astigmata	Acaridia	Carpoglyphidae	<i>Carpoglyphus lactis</i>	46	88	
		Glycyphagidae	<i>Lepidoglyphus destructor</i>	5	8	
	Acaridae		<i>Acarus siro</i>	5	93	
			<i>Acarus gracilis</i>	9	28	
			<i>Aleuroglyphus ovatus</i>	6	90	
			<i>Tyrophagus putrescentiae</i>	100	100 *1	
			<i>Tyrophagus brevicrinatus</i>	58	73	
			<i>Caloglyphus redickoverzi</i>	7	9	
	Psoroptidia	Pyroglyphidae		<i>Dermatophagoides farinae</i>	6	5
				<i>Dermatophagoides pteronysinus</i>	5	6
Prostigmata		Cheyletidae	<i>Cheyletus malaccensis</i>		7	
			<i>Ephestia kuehniella</i> larvae	5	5	
			<i>Tribolium castaneum</i> larvae	5	5	
			<i>Cheyletus malaccensis</i> feed on <i>T. putrescentiae</i>		49 *2	
			mite's diet	5	6	
			wheat kernels	5	6	

Anti- *T. putrescentiae* antibodies cross-react partially with mites of Acaridae and Carpoglyphidae family. No cross-reaction was found out for family Glycyphagidae (*Lep. destructor*, *Cal. redikorzevi*), stored product insects, yeast and wheat kernels. The detection threshold was 2.2µg of mites/ml. PTA-ELISA detected 6 individuals of *T. putrescentiae* per 0.5ml of homogenization buffer

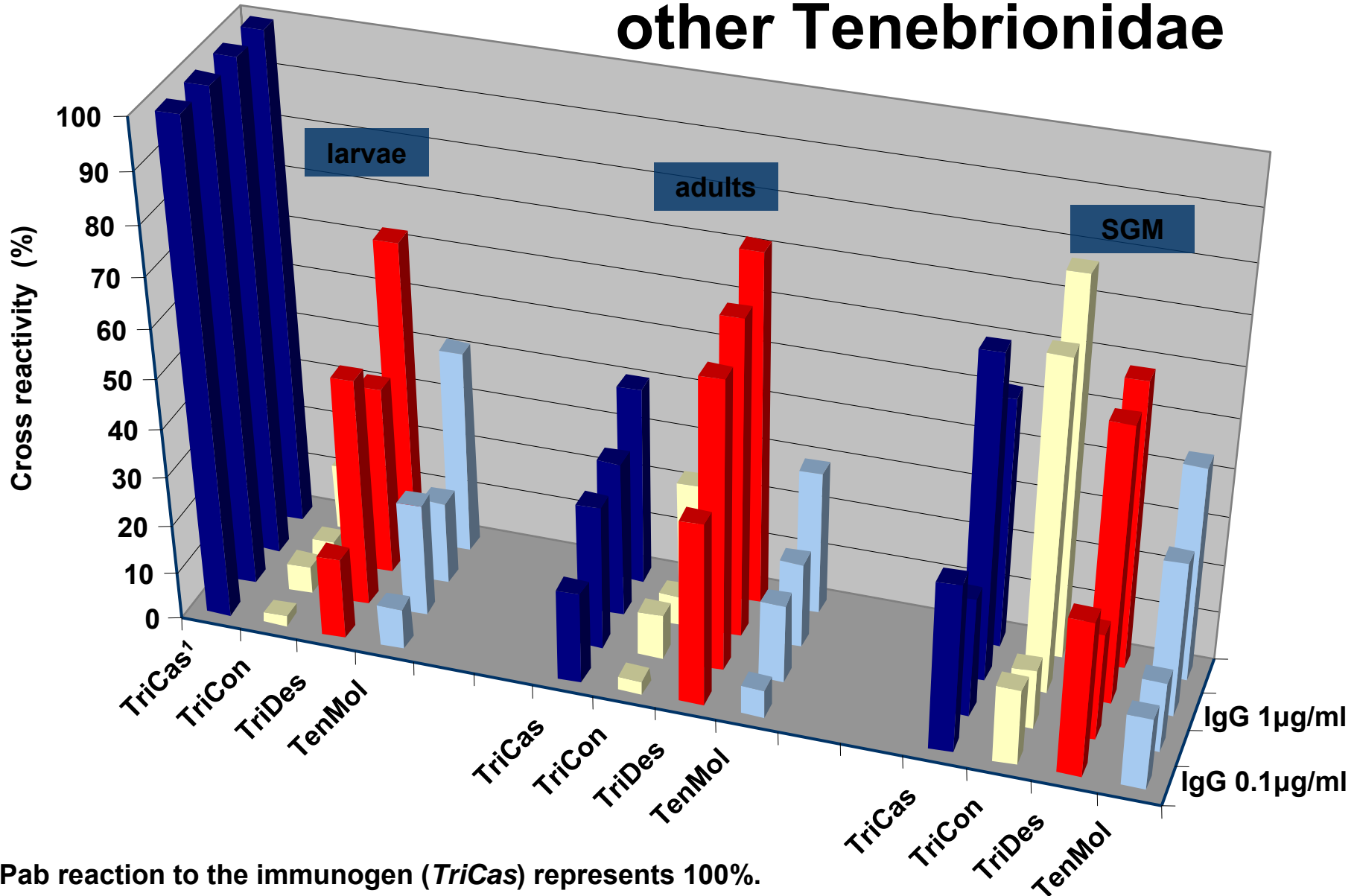
Specificity of anti-*A. siro* Abs

		Relative assay response (%) after			
		15 minutes		30 minutes	
		1µg	10µg	1µg	10µg
	incubation time concentration				
immunogen	<i>Acarus siro</i> *	100	100	100	100
beetles	<i>Tribolium castaneum</i> larvae *	0	0	2	4
	<i>Tribolium castaneum</i> larvae	1	1	2	3
	<i>Tribolium castaneum</i> adults	0	0	0	0
	<i>Sitophilus granarius</i>	0	0	1	1
moths	<i>Ephestia kuehniella</i> larvae	0	0	1	2
	<i>Ephestia kuehniella</i> larvae *	1	1	1	3
mites	<i>Aleuroglyphus ovatus</i> *	1	1	4	6
	<i>Aleroglyphus ovatus</i> faeces	1	2	4	6
	<i>Caloglyphus redickoverzi</i> *	2	3	9	12
	<i>Caloglyphus redickoverzi</i> faeces	1	2	6	9
	<i>Tyrophagus putrescentiae</i> *	1	1	3	8
	<i>Tyrophagus putrescentiae</i>	0	1	4	8
others	mite's diet	0	0	0	0
	wheat kernels	0	1	0	0
	semolina	0	0	1	1



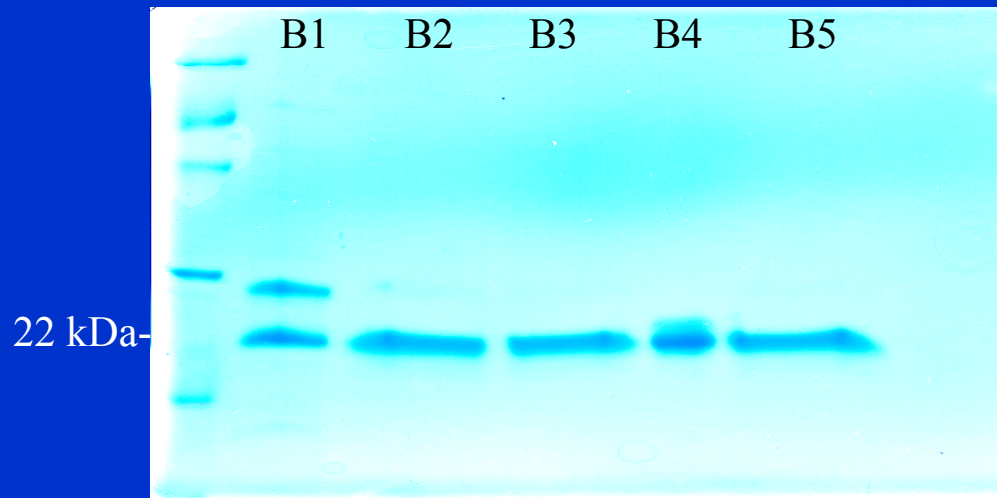
Detection threshold = 3 mites

Cross reactivity of *anti-TriCas* K51 to other Tenebrionidae



KUNITZ-TYPE PROTEASE INHIBITOR CHIPS

Production of recombinant His-tagged KPI proteins

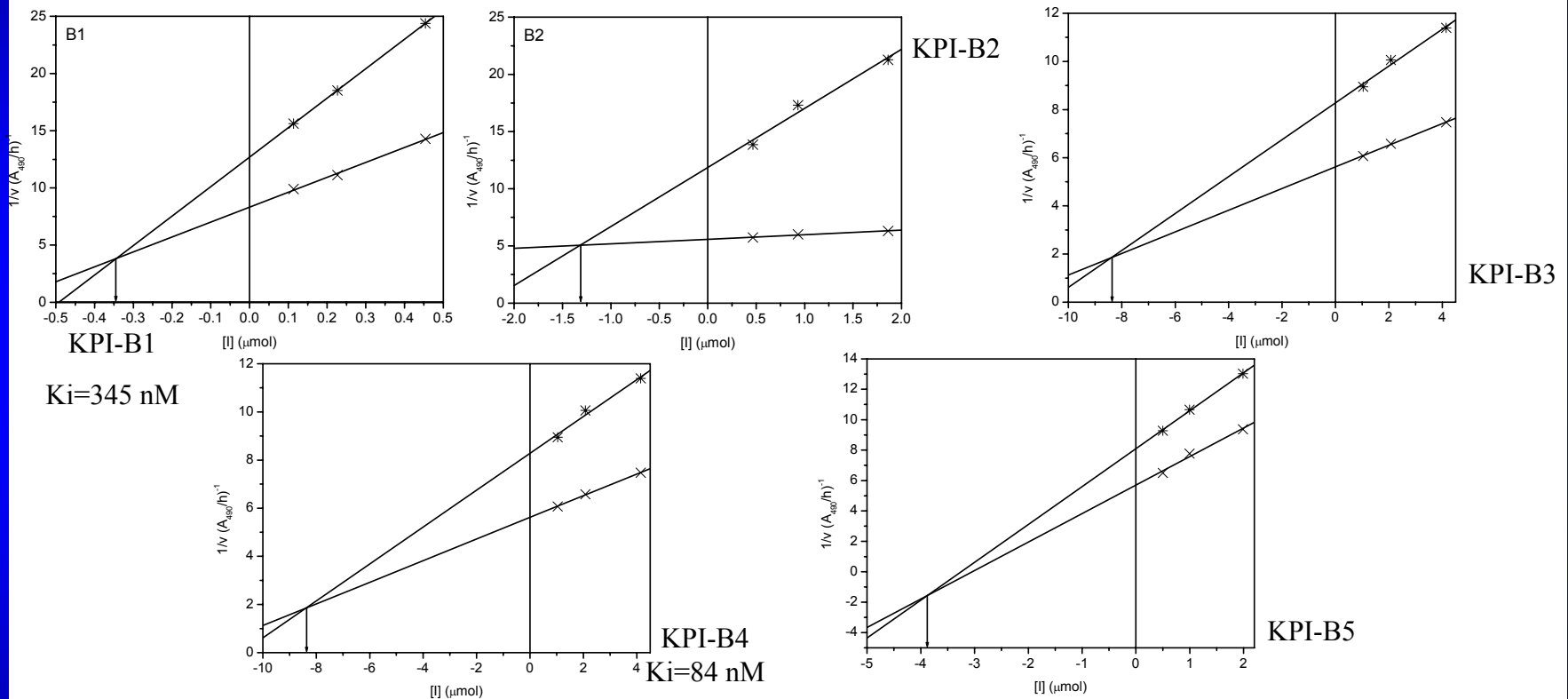


Expression of 2 KPI-A (A1, A2) cathepsin D/ trypsin Inhibitors

Expression of 5 KPI-B (B1 to B5) trypsin/ chymotrypsin Inhibitors.

Expression of 6 KPI-C (C1 to C6) cathepsin B/ bromelain Inhibitors.

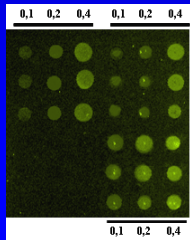
Kunitz-type protease inhibitors are effective in blocking insect and mite trypsins



Inhibition of bovine trypsin activity by Kunitz-type competitive protease inhibitors B1, B2, B3, B4, B5. Inhibition kinetic data shown in Dixon-plot $1/v$ as a function of $[I]$ for a competitive inhibitor at two different substrate concentrations of BANA \square 0.125 mM - 0.250 mM. The arrow point shows $-K_i$. **KPI-B2 ($K_i = 1,3 \mu\text{M}$), B3 ($K_i = 8,3 \mu\text{M}$ and B5 ($K_i = 3,8 \mu\text{M}$), weak inhibitors of bovine trypsin, have insect/mite anti-trypsin activity.**

KUNITZ-TYPE PROTEASE INHIBITOR CHIPS

APPLIED TO PROTEASE DETECTION



Chip Miniaturization:

1 nl spots produced by SpotArray (Perkin Elmer)
high spot density

sub-arrays for multiple hybridisations

0,2 ng/nl minimum amount of KPI in each spot

Stability of KPI proteins at 4 °C

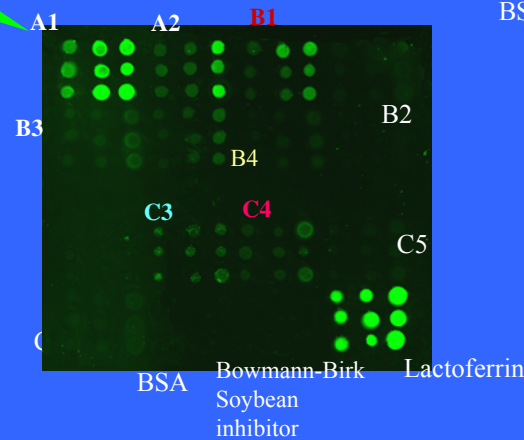
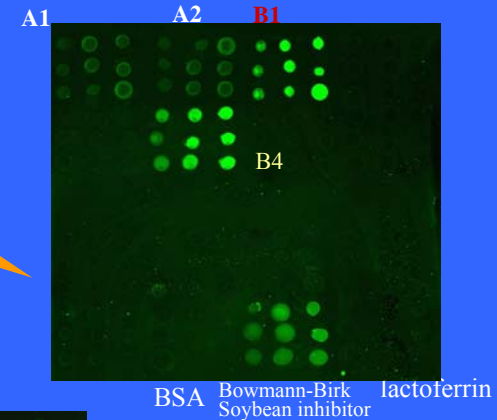
Three PKIs, group A, B and C, immobilized onto a surface, were tested in the efficiency of binding at decreasing trypsin (10-100 ng/ μ l) concentrations. High sensitivity of detection by KPI-A was put in relation to highest stability of KPI-A bound to the glass.

Protein diversity on the array surface is studied using saturation binding curves for each protease-protein inhibitor interaction, using multiple concentration points, to evaluate binding independently of the amount and density of active protein bound in each spot (Mc Beath, J. Am. Chem. Soc. 2006).

KUNITZ-TYPE PI ARRAYS

Interaction with serine proteases

Interaction with cysteine proteases



A: cathepsin D/ trypsin Inhibitors

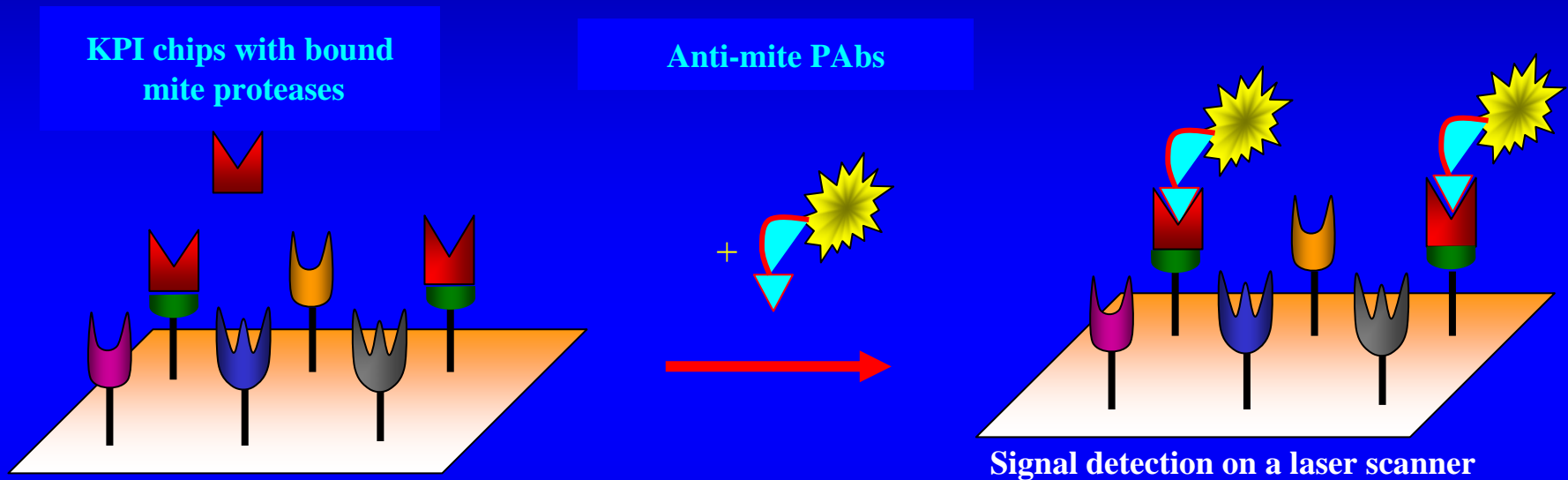
B: trypsin/ chymotrypsin Inhibitors

C: cathepsin B/ bromelain Inhibitors

Group A, group B and group C Kunitz-type PI-arrays were hybridized with a solution containing bovine trypsin or cathepsin B (labeled with Alexa-555).

Protein chips in the detection of mite proteases

Detection on-chip by anti-mite polyclonal Abs



Mite / insect allergens spread in human environments and stored foods, with trypsin, chymotrypsin, aspartic and cysteine protease allergens highly abundant in spent growth media (SGM) and feces.

Direct detection, using labeled polyclonal Abs (*Acarus siro*, *L. destructor*, *T. putrescentiae*)

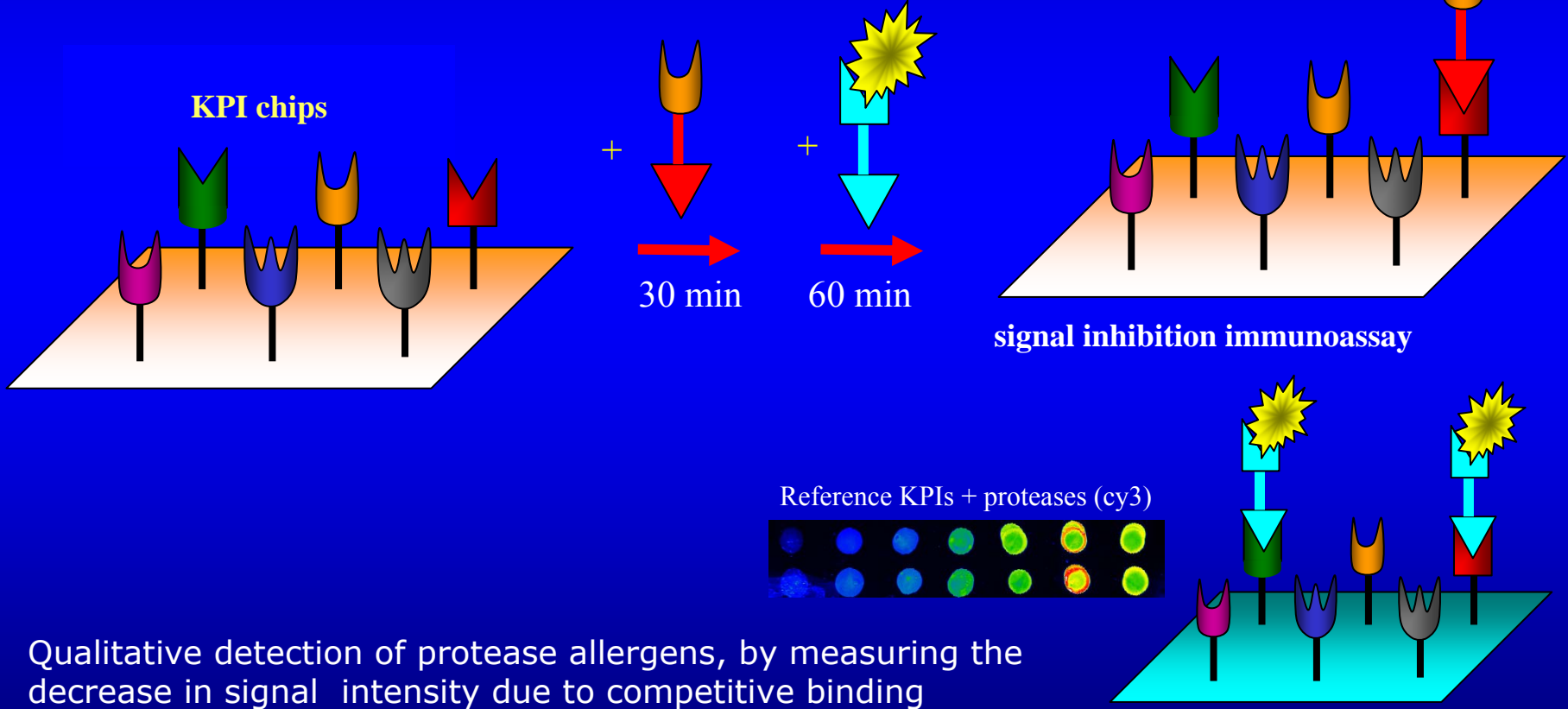
Detection of protease allergens, with discrimination at species, or family level

Polyclonal antibodies need purification (ProteinG column) before labelling with Alexa-555.

Protein chips in the detection of mite proteases

Detection by signal displacement

a) Hybridization with mite/insect extracts b) Addition of cy3-proteases decrease in protease binding



Qualitative detection of protease allergens, by measuring the decrease in signal intensity due to competitive binding

It is not useful in species discrimination (mixed populations).

UNDER DEVELOPMENT

Advantages of protein chips:

Good sensitivity

Good assay range

Working range are larger than that of ELISA tests

Signal/noise ratio is better than that of ELISA tests

Discriminatory power at low concentrations is superior

Protein chips testing may be transferred to biosensor formats

Disadvantages:

Fluorescence labeling, and need of a scanner:

Biosensors may use direct detection methods

CONCLUSIONS

Physical interaction between KPIs and proteases were studied on chips either with the first or the second being in solution

There is a limit in chip technology when comparing proteins on the surface and their binding ability to a protein partner at one concentration point.

Binding to surface reduces the amount of active protein. Comparative evaluation of proteins at different concentrations of protease partner was made, to assess individual KPI efficiency in protease binding, depending on the real amount of active protein bound to the surface.

KPI-A proteins bound to chip surface performed best than all other KPI groups. The use on the same chip of KPI-A, -B and -C proteases allows protease detection in a large range of sample concentrations

Mite protein extraction from some foods (flours) is a critical point for testing

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