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Characterization of Kunitz-type inhibitor B1 performance using protein chips and AFM

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ABSTRACT

A protein chip containing 11 different proteases was used to investigate the expression of protease inhibitors induced by *Aspergillus carbonarius* infection of potato tubers. Kunitz-type protease inhibitors B1 (KPI-B1) recombinant proteins with trypsin/chymotrypsin protease selectivity were used as internal control and inter-assay control in protease chips targeting KPI proteins. KPI-B1 was spotted on each chip as a reference to compare fluorescence intensities between different hybridization experiments. In order to validate the use of KPI-B1 as control, we studied the performance of recombinant KPI-B1 protein. Interactions between KPI-B1 and proteases in the absence and in the presence of phenylmethylsulfonyl fluoride (PMSF) indicated that a free substrate binding pocket in protease is required for binding with the recombinant KPI-B1 inhibitor. Atomic force microscopy (AFM) was employed to analyse structures of protein complexes formed by KPI-B1. KPI-B1/antibody complexes have diameters of ca. 450 nm and heights of ca. 8 nm, while trypsin/KPI-B1/antibody complexes have relatively small diameters (ca. 300 nm) but very great heights (ca. 50 nm). On the basis of AFM data, trypsin-KPI-B1 complexes, instead of KPI-B1 alone, could be a better internal control for protein chip to calibrate fluorescence signals obtained from different hybridization experiments.

1. Introduction

Kunitz-type protease inhibitors (KPIs) with size of 22–24 kDa are a class of plant protease inhibitors, found in seeds, storage organs and vegetative tissues. They play an important role in plant defence against pathogens and insects [1,2]. The Kunitz-type inhibitors found in the potato and other *Solanum* species are divided into three homology groups, A, B and C. The KPI-A group includes inhibitors of aspartic proteases and chymotrypsin, the KPI-B group contains dual inhibitors of the serine proteases, trypsin and chymotrypsin, and the KPI-C group includes inhibitors of plant cysteine proteases as well as invertase [3]. Some KPIs play important roles in biotic stress response, but little is known about induced KPIs' specificities toward different proteases.

Recently, 11 sequences of KPIs from *Solanum palustre*, a non-tuberous South-American species belonging to the subsection *Estolonifera* in the section *Petota*, were deposited in Genbank. Five of them were further characterized, of which KPI-B1 and B4 showed optimum Ki in nanomole range (84.8 nM for B4 and 345.5 nM for B1) [1].

Over the past few years, protein chip-based technologies have arisen as a powerful tool for discovering protein functions and protein–protein interactions. This technology allows fast, easy and parallel detection of multiple addressable elements with minimum amount of sample in a single experiment [4]. Protein immobilization is an important step to produce protein chips. The covalent binding of NH_2– residues to epoxy-activated slides enhances the stability of immobilized proteins but may cause changes in protein conformation after immobilization resulting in modification of protein active site accessibility, or changes in the protein microenvironment due to the interaction between the support materials and the protein. Therefore, an optimization of method in the efficiency of protein immobilization on the slide surface regarding functional conservation and reproducibility of the amount of immobilized protein is needed. Since there are numerous factors in protein chip manufacture and utilizing could affect the fluorescent intensity, a serial of control spots could be very helpful to compare inter-assay results obtained with multiple chips hybridizations.

KPI-B1 has been used for its potential biotechnological applications. There is an up to 99% high level amino acid sequence identity between KPI-B proteins from cultivated potato and *S. palustre*. Therefore, it is possible to detect the *S. palustre* KPI (Spls-KPI)

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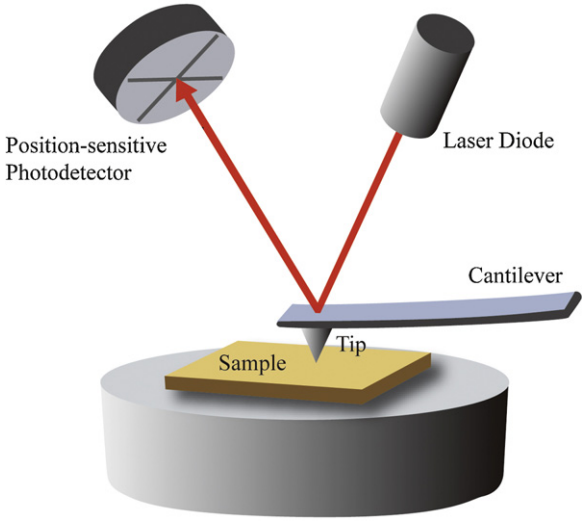

Fig. 1. Diagram of AFM work principle.
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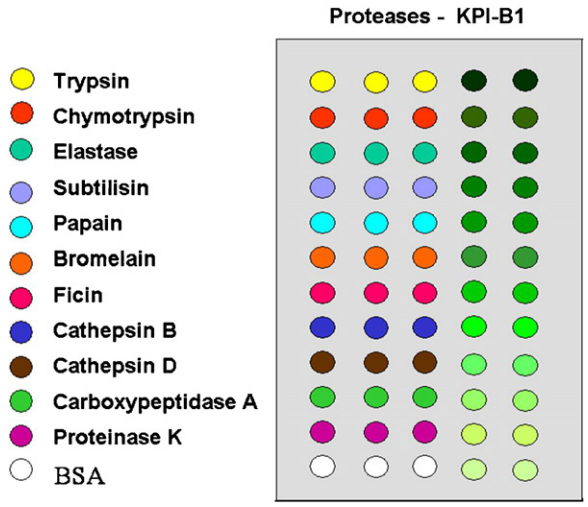

Fig. 2. Schematic diagram of the protein chip containing 11 proteases for detecting fungal infection.

proteins using a polyclonal antibody recognizing cultivated potato KPI proteins. A protein chip made of four recombinants, i.e., KPI-B1 and B4 from group B, and KPI-A1 and A2 from group A, was used to capture and detect mite proteases. Spls-KPI-B1 showed the highest capturing and detecting abilities [5].

Atomic force microscopy (AFM) is a powerful tool, which has been widely used in the areas of biology, nanotechnology, and other fields during the past two decades [6,7]. It can image samples on a nanometer scale with minimum or even no preparation. The basic idea of AFM is to use a sharp tip to sense the surface of a sample. During the test, a laser diode emits a laser beam onto the back of the cantilever over the AFM tip. The angular deflection of the reflected laser beam, which is changing with the change of the interaction between the tip and the sample, is detected by a position-sensitive photodiode (Fig. 1). AFM has been successfully applied to fish gelatine characterizing [8], microorganism detection [9], and protein structure monitoring [10]. It is also a suitable tool to analyse the physical structures of protein and protein complexes.

In the present work, a protein chip containing 11 proteases was prepared to detect fungi infection of potato tuber. Recombinant Spls-KPI-B1 was used as a control on the chip to compare fluorescent signals originated from potato tuber KPIs induced during a 72-h course of *Aspergillus carbonarius* infection. A protein chip protocol was set up. The potato KPIs were hybridised to the protease chip to quantify their level of expression. The specificities of KPIs with different protease (toward different serine and cysteine proteases of the same group) were also investigated.

In order to validate the use of KPI-B1 as internal control on protease chips, to visualize the structure of KPI-B1 bound trypsin and to study whether the binding to the polyclonal antibody was affected by different KPI-B1 structural organization, AFM was employed to characterize structures of KPI-B1 and its protein complexes with trypsin and with anti-KPI antibodies.

2. Methods

2.1. Preparation of KPI-B1

Polyclonal antibody recognizing potato KPIs was kindly provided by Dr. A. Shevelev (INBI, Bach Institute, Moscow, Russia). Recombinant KPI-B1 was produced and purified as described by Speransky et al. [1]. Briefly, the KPI-B1 was expressed in *Escherichia coli* using the pQE30 vector (Qiagen, Hilden, DE). Cultures (100 ml) of *E. coli* strain BL21(DE3) transformed with expression plasmids were grown at 37 °C to A_{600} 0.5–0.6 with shaking at 200 rpm. They were then transferred and induced with IPTG (1 mM) at 25 °C for 24 h. Cells were harvested by centrifugation at 5000 × *g* and the pellet frozen at −80 °C. Cells were then extracted with 50 ml Tris–HCl buffer (50 mM) with benzonase (pH 8.0, Bugbuster, Novagen, Merck Biosciences, Darmstadt, DE). After extracting for 30 min at room temperature, the homogenates were centrifuged at 16,000 × *g* for 20 min at 4 °C, and the clear supernatant was applied to a His select cartridge column (1.25 ml). Elution was carried out according to the manufacturer's instructions (Sigma-Aldrich, USA). The eluted product was analysed with SDS–PAGE and the gel was stained with Blue Coomassie. One band with a MW of 21 kDa corresponding to the Kunitz-type inhibitors was visualized.

2.2. Manufacture of protein chip with six proteases for protease binding pocket study

Each of trypsin, chymotrypsin, cathepsin B, papain, subtilisin and elastase with a concentration of 0.5 μg/μl in 0.1 M $NaHCO_3$ buffer (pH 9) with 40% glycerol was spotted in triplicate onto epoxysilane-coated Nexterion slides (Schott, Jena, Germany) using

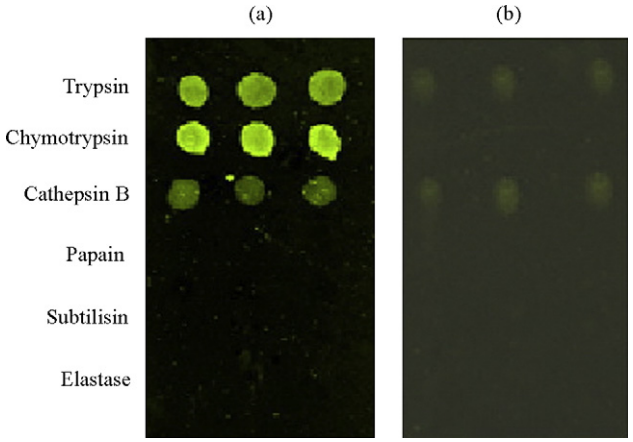

Fig. 3. Hybridization of fluorescent KPI-B1 on the protease chips containing trypsin, chymotrypsin, cathepsin B, papain, subtilisin and elastase. (a) KPI-B1 binding to trypsin, chymotrypsin and cathepsin B. (b) After PMSF treatment.

a robotic printing SpotArray 24 system (Perkin Elmer, Waltham, MA, USA) [4,11]. The proteins were immobilized by the covalent binding between the epoxy groups on the slide surface and the amine groups in the proteins. The printed slides were incubated in dark at room temperatures and 60% humidity for 2 h to allow the residual epoxy groups reacted with water. Then, the printed slides were kept dry in dark until use.

Hybridization was performed with (recombinant) Spls-KPI-B1 and a control, soybean trypsin–chymotrypsin Bowman–Birk inhibitor (SB-TI) (SIGMA, St. Louis, USA). Both of the inhibitors were labelled withAlexa-555 protein labeling kit (Molecular Probes, Life Technologies, Carlsbad, CA, USA). Biogel P-6 (Bio-Rad, Hercules, CA, USA) was used to remove excessive free dye as described previously [1]. The protein chips then were incubated on a shaker at room temperature for 1 h in 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 drained in a centrifuge at 500 × *g* for 2 min. To investigate the effects of phenylmethylsulfonyl fluoride (PMSF) on blocking active pockets of the proteases, chips spotted with the sample proteases were treated with 50 mM Tris buffer pH 7.4 used as a ten-fold dilution of 10 mg/ml PMSF in methanol. The chips were then hybridised with the two inhibitors through the same procedure after wash.

2.3. Preparation of protease chip containing 11 proteases for detection of fungal infection

Trypsin, chymotrypsin, elastase, subtilisin, papain, bromelain, ficin, cathepsin B, cathepsin D, carboxypeptidase A and proteinase K (Sigma, St. Louis, USA), dissolved at 1 mg/ml in 0.1 M $NaHCO_3$ (pH 9), were spotted in triplicate to make a protease chip. The scheme of the chip is illustrated in Fig. 2. The chip was used to monitor the KPI inhibitors induced during fungal (*A. carbonarius*) infection of tuber slices. Differences among protease inhibitors were investigated before the infection and 24, 48 and 72 h after the infection occurred, respectively. The potato proteins were extracted using 50 mM Tris/Tween-20 buffer (pH 7.4). The extracts were hybridised on the protease chip, followed by the detection of KPIs using the anti-KPI antibodies labelled with fluorescence using the Alexa-555 protein labeling kit. The remained free dye was removed with Biogel P-6 (Bio-Rad, Hercules, CA, USA) gel-filtration.

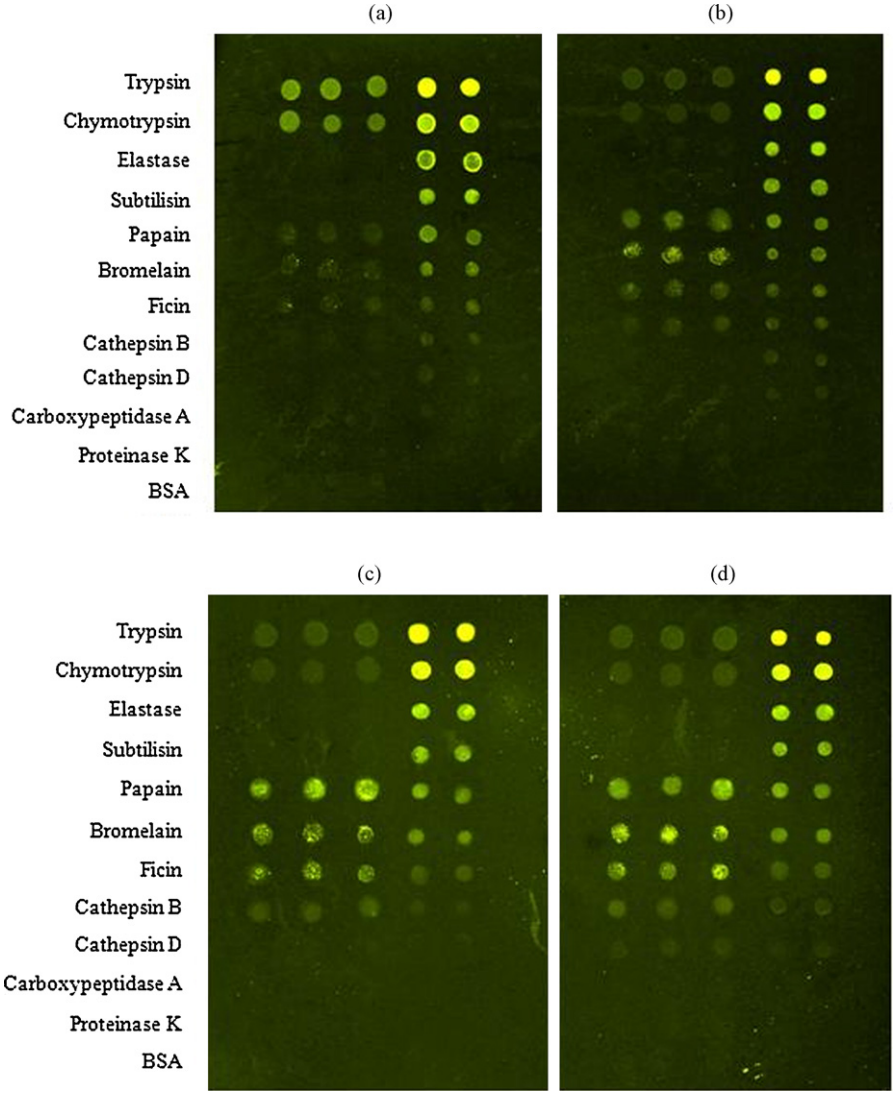

Fig. 4. Application of the protein chip containing 11 proteases in the monitoring of the expression of KPIs with different protease specificities in potato tuber before and during *A. carbonarius* infection. Fluorescent anti-KPI antibodies were used for detection. (a) Uninfected potato, control extract; (b), (c) and (d) potato extracts after 24, 48 and 72 h of infection, respectively. KPI-B1 spots (the last two columns on each chip), hybridised with fluorescent anti-KPI antibodies, were used to calibrate fluorescence signals on different protein chips. KPI-B1 dose (from top to bottom): 9000, 4500, 2250, 1125, 562, 281, 140, 70 and 35 pg/spot.

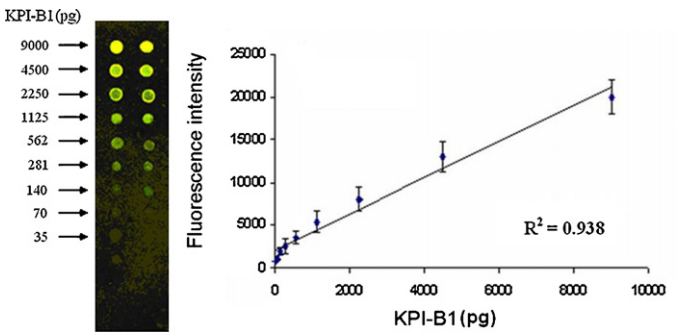

Fig. 5. Linear relationship between fluorescence intensity and dose of KPI-B1s hybridised with fluorescent labelled anti-KPI antibodies.

Serially diluted KPI-B1 was spotted in duplicate on the chip to form a sub-array (from 1500 to 133 pg KPI-B1 per spot). The polyclonal antibody recognizing KPIs was bound to the sub-array, which was used as a control to compare the different hybridization experiments. The aforementioned hybridization methods were used. Experiments were performed in triplicate and replicated on different days. The obtained fluorescence intensities were analysed with software StatView 4.01 (Abacus Concept Inc.). Differences between groups were analysed using the Student's *t*-test. Differences between repeated measures were tested using variance analysis (ANOVA) and Fisher's protected least squares difference test at a 0.05 level of significance. These analyses were performed with software StatView 4.01 (Abacus Concept Inc.).

2.4. Preparation of AFM samples

To obtain a protein devoid of salt, suitable for AFM analysis, the KPI-B1 with a concentration of 1 mg/ml (determined before buffer change) was desalted with Zeba desalt spin columns (Pierce, Rockford, IL, USA) and rinsed twice with MilliQ grade H_2O. The same procedure was used to desalt the polyclonal antibody and trypsin. Two types of protein complexes, KPI-B1 + polyclonal antibody and KPI-B1 + trypsin + polyclonal antibody, were prepared. To form the KPI-b1 + polyclonal antibody complex, a KPI-B1 aliquot was incubated with the polyclonal antibodies for 30 min. To prepare the KPI-b1 + trypsin + polyclonal antibody complex, an aliquot of KPI-B1 was incubated with trypsin for 30 min first. Then, the products were incubated with the antibodies for another 30 min. AFM samples were prepared by spotting a small aliquot of the diluted protein or protein complex solution directly on a freshly-cleaved mica surface. The mica surface was then dried by forced air using an ear syringe before the AFM investigation.

2.5. AFM investigation

The nanostructure investigation was performed using a Nano-R2™ AFM (Pacific Nanotechnology, Inc., Santa Clara, CA, USA) with noncontact mode in air under ambient temperatures. An NSC11/noAl tip (radius of the curvature <10 nm, full tip cone angle <30°, MikroMasch, Wilsonville, OR, USA) with a force constant of ca. 48 N m^{-1} and a resonant frequency of ca. 340 kHz was used. The noncontact mode in this type of AFM is similar to the intermittent contact mode commonly mentioned in other AFM equipment. The scanning rate ranged from 0.5 to 2.0 Hz. Image analysis was carried out offline with the use of AFM software (NanoRule+ 2.0).

3. Results and discussion

3.1. Studies of the protease binding pocket in recombinant KPI-B1 protease inhibitors

Protein chips containing six proteases (trypsin, chymotrypsin, cathepsin B, papain, subtilisin and elastase) were used to investigate interactions between KPI-B1 and these proteases in the

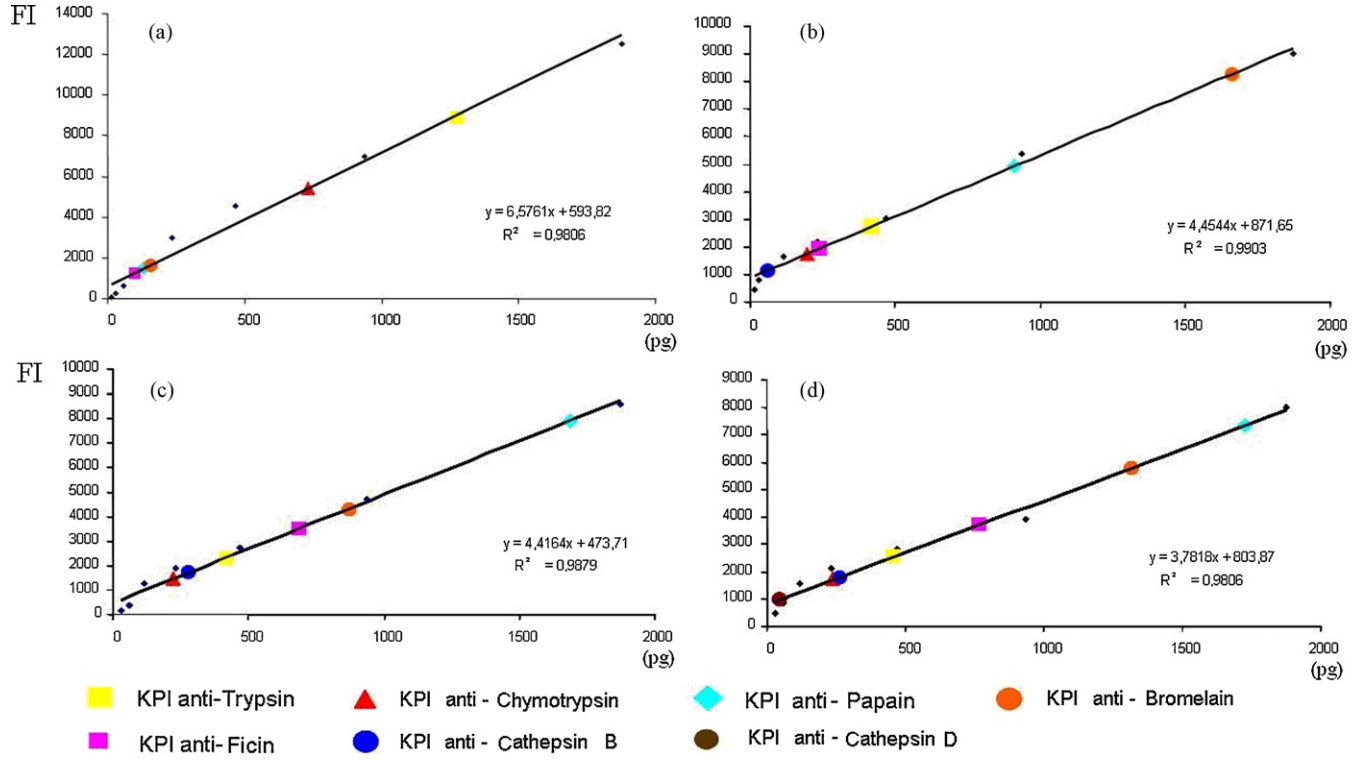

Fig. 6. Fluorescence intensities of KPI-B1 sub-arrays on protease chips after hybridizing with potato extracts. (a) Control, potato extract before fungal infection; (b), (c) and (d) potato extracts after 24, 48, and 72 h of fungal infection, respectively.

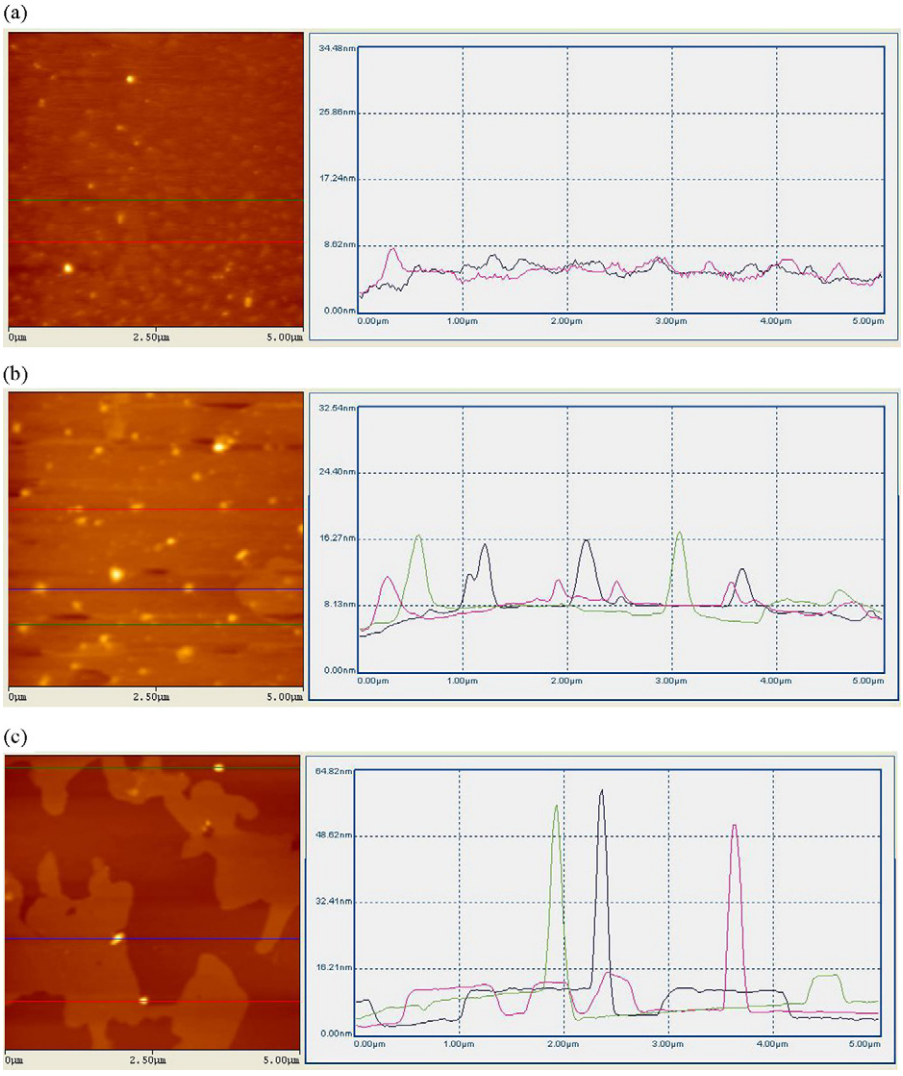

Fig. 7. AFM images and line analyses of (a) KPI-B1, (b) complex formed by KPI-B1 bound to polyclonal antibodies and (c) complex formed by KPI-B1 bound to trypsin and polyclonal antibodies.

absence and in the presence of PMSF. KPI-B1, (as already observed for SB-TI, data not shown), could bind to trypsin, chymotrypsin and cathepsin B in the absence of PMSF (Fig. 3(a)). However, KPI-B1, as also observed for SB-TI, could not bind to any of these six proteases after the PMSF treatment which blocks the active pockets of the proteases (Fig. 3(b)). The results indicated that the recombinant KPI-B1 inhibitor behaves in protease inhibition as the native KPI-B proteins, interacting with the free substrate binding pocket in proteases.

3.2. Detection of fungal infection with protease chip containing 11 proteases

In potato tuber, several KPIs with different protease specificities could be induced by fungal and nematode infections [12,13]. To assess their specificities and the induction times of each type of KPI, a protein chip containing 11 proteases was prepared. Tuber extracts before and after 24, 48 and 72 h of *A. carbonarius* infection were investigated with the chip (Fig. 4).

At least seven KPIs showing specific protease-specificities were induced during the tuber infection. Maximum fluorescence intensities obtained with bromelain, ficin, cathepsin B and cathepsin D were detected after 72 h of infection. However, the fluorescence intensities obtained with trypsin and chymotrypsin became lower after the infection, which could be explained by competitive binding of other inhibitors co-induced during the infection. The competitive binding of protease inhibitors with different sizes is known for trypsin/chymotrypsin specific potato protease inhibitors (PPIs). It has been reported that Potato I and Potato II small inhibitors with size of 10 kDa were induced during a pathogen (*Botrytis cinerea*) infection of potato tuber [14]. KPIs and PPIs specific to trypsin/chymotrypsin probably were both induced during the *A. carbonarius* infection. Since a large portion of trypsin/chymotrypsin could be bound to PPIs, they became not available to interact with KPIs, causing the huge decrease of fluorescence intensities obtained with trypsin and chymotrypsin after the infection. Meanwhile, a slight decrease in fluorescence intensity obtained with papain after 48 h of the infection was detected. It might be due to competitive binding of other protease inhibitors with papain specificity induced after 48 h. Another possible hypothesis of the slight decrease might be a decrease of the induction of papain-specific KPI mRNA after 48 h.

KPI-B1 can be used as positive control in protein chip experiments to compare fluorescent signal on different chips when the same anti-KPI antibody was used. In this work, a control sub-array composed of serially diluted KPI-B1 spots was included on each

protease chip (Fig. 4). The Anti-KPI antibody used for hybridization was successfully tested with potato KPI proteins having 90% sequence identity. And KPI-B1 possesses 94.5% amino acid identity with the potato protein KPI-B10 used in rabbit immunization. Since the polyclonal antibodies could bind to KPI-B1 at several binding sites, the fluorescence intensity of the KPI-B1 sub-array is stronger than that obtained from the KPIs induced by the *A. carbonarius* infection. The KPI-B1 sub-array allowed visualization of positive hybridization and determination of inter-assay variability of fluorescence intensity (Figs. 5 and 6). The KPI-B1 control provided a calibration curve of fluorescence intensity versus KPI-B1 dose (Fig. 5). A good linear relationship between them was observed. Normalization of fluorescence intensities at each experimental point (before the fungal infection and at 24 h, 48 h or 72 h after the infection) could be achieved by comparing the calibrations of the fluorescence intensities of the KPI-B1 sub-arrays (Fig. 6). The results indicated that the expression in potato tuber of different protease-specific KPIs increased during the infection with a profile characteristic of each KPI type.

KPI-B1 could be a valuable control for inter-assay fluorescence intensity analysis. In order to validate its potential, the efficiencies and reproducibility of KPI-B1 binding to anti-KPI antibodies, proteases binging to the chip, hybridising with the KPI in the potato extracts, and their subsequent detection by the polyclonal antibodies under the experimental conditions will be investigated in our future work.

3.3. AFM analysis

Atomic force microscopy was employed to analyse the sizes and structures of KPI-B1 and its two protein complexes: KPI-B1 + polyclonal antibodies, and KPI-B1 + trypsin + polyclonal antibodies. The bright areas in the AFM images (left in Fig. 7) corresponded to particles on sample surface. The heights and diameters of the particles were determined with line analysis of the images (right in Fig. 7). The free KPI-B1 particles with diameters of ca. 250 nm and heights of ca. 3 nm were observed in Fig. 7(a). After KPI-B1 binding to the polyclonal anti-KPI antibodies, particles with diameters of ca. 450 nm and heights of ca. 8 nm were formed (Fig. 7(b)). When KPI-B1 was bound with trypsin and the polyclonal antibodies to create trypsin/KPI-B1/antibody complexes, particles with smaller diameters (ca. 300 nm) but much greater heights (ca. 50 nm) were observed (Fig. 7(c)).

The AFM images indicated that free KPI-B1 has a compact (molten, globule-type) structure. However, when KPI-B1 bound to trypsin, it changed into a filamentous and elongated shape, which only offered binding epitopes to a subset of anti-KPI antibodies. Since their sequences are highly similar to each other, this conformational change should also occur in the potato tuber KPI. Thus, it should be possible to use KPI-B1 bound to trypsin as the internal control in protease chips, making this tool more suitable for comparison of multi-chip hybridization experiments. The proteases on the chip could bind to inhibitors, then visualized and detected by the polyclonal antibodies. In our future work, control sub-arrays formed with trypsin-bound KPI-B1 will be evaluated, and the results will be compared with those obtained in this work.

4. Conclusions

Protein chips were successfully used to investigate the expression of protease inhibitors induced by *A. carbonarius* infection of potato tubers. The results indicated that at least seven KPIs showing specific protease-specificities were induced during the infection. The fluorescence intensities of the hybridization experiments were quantitatively compared by using KPI-B1 recombinant proteins as internal control and inter-assay control. The investigation of interactions between KPI-B1 and proteases in the absence and in the presence of PMSF indicated that a free substrate binding pocket in protease is required for binding with the recombinant KPI-B1 inhibitor. AFM images revealed that KPI-B1 changed into a filamentous and elongated shape when it bound to trypsin, which might only offer binding epitopes to a subset of anti-KPI antibodies. Therefore, trypsin-KPI-B1 complexes, instead of KPI-B1 alone, could be a better internal control for protein chip to calibrate fluorescence signals obtained from different hybridization experiments.

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References

[1] A.S. Speransky, F. Cimaglia, A.A. Krinitsina, P. Poltronieri, P. Fasano, A.M. Bogacheva, T.A. Valueva, D. Halterman, A.B. Shevelev, A. Santino, Kunitz-type protease inhibitors group B from *Solanum palustre*, Biotechnology Journal 2 (2007) 1417–1424.
[2] T.A. Valueva, V.V. Mosolov, Role of inhibitors of proteolytic enzymes in plant defence against phytopathogenic microorganisms, Biochemistry (Moscow) 69 (2004) 1305–1309.
[3] A. Heibges, F. Salamini, C. Gebhardt, Functional comparison of homologous members of three groups of Kunitz-type enzyme inhibitors from potato tubers (*Solanum tuberosum* L.), Molecular Genetics and Genomics 269 (2003) 1215–1221.
[4] M.F. Templin, D. Stoll, J.M. Schwenk, O. Pötz, S. Kramer, T.O. Joos, Protein microarrays: promising tools for proteomic research, Proteomics 3 (2003) 2155–2166.
[5] P. Poltronieri, F. Cimaglia, A. Santino, M.D. De Blasi, I. Krizkova-Kudlikova, S. Liu, Y. Wang, Y. Wang, Protein chips for detection of mite allergens using Kunitz-type protease inhibitors, Biotechnology Journal 5 (2010) 582–587.
[6] Y.F. Dufrene, AFM for nanoscale microbe analysis, Analyst 133 (2008) 297–301.
[7] S. Liu, Y. Wang, Application of AFM in microbiology: a review, Scanning 32 (2010) 61–73.
[8] Y. Wang, H. Yang, J.M. Regenstein, Characterization of fish gelatine at nanoscale using atomic force microscopy, Food Biophysics 3 (2008) 269–272.
[9] H. Yang, Y. Wang, Application of atomic force microscopy on rapid determination of microorganisms for food safety, Journal of Food Science 73 (2008) N44–N50.
[10] T. Wangkam, T. Srikhirin, P. Wanachantararak, V. Baxi, B. Sutapun, R. Amarit, Investigation of enzyme reaction by surface plasmon resonance (SPR) technique, Sensors and Actuators B: Chemical 139 (2009) 274–279.
[11] G. MacBeath, S.L. Schreiber, Printing proteins as microarrays for high-throughput function determination, Science 289 (2000) 1760–1763.
[12] G. Bauw, H.V. Nielsen, J. Emmersen, K.L. Nielsen, M. Jørgensen, K.G. Welinder, Kunitz protease inhibitors and other major proteins in tuber of potato cv. Kuras, FEBS Journal 273 (2006) 3569–3584.
[13] D. Turrà, D. Bellin, M. Lorito, C. Ghebardt, Genotype-dependent expression of specific members of potato protease inhibitor gene families in different tissues and in response to wounding and nematode infection, Journal of Plant Physiology 166 (2009) 762–774.
[14] M.R. Hermosa, D. Turra, V. Fogliano, E. Monte, M. Lorito, Identification and characterization of potato protease inhibitors able to inhibit pathogenicity and growth of *Botrytis cinerea*, Physiological and Molecular Plant Pathology 68 (2006) 138–148.

Biographies

Palmiro Poltronieri received his Ph.D. degree from Institute of Biochemistry at Verona University in 1995. He has been a Research Scientist at Institute of Sciences of Food Production, Unit of Lecce, since 2001. His researches focus on protein reactions and applications of protein chip as well as DNA array.

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Fabio Cimaglia obtained his Ph.D. degree in nanotechnology from Italian Institute of Technology in 2007. His research focuses on methods to apply protein chips and other advanced biotechnologies on detection of allergens, bacterial pathogens and disease biomakers. Currently, he is under contract with a small medium Enterprise, Biotecgen srl, in Lecce.

Angelo Santino received his Ph.D. degree in Biological Science at the University of Lecce in 1988. He has worked at Institute of Sciences of Food Production, Unit of Lecce, since 1994. He also shortly worked as visiting scientist in John Innes Centre, Norwich, UK, Institut de Biologie Moleculaire des Plantes-CNRS, Strasburg, France, and Institut des Sciences du Vegetal, CNRS, Gif sur Yvette, France. His main research interests are in development of innovative technologies and biotechnologies for bio-medical and agro-food applications of plants.

Yifen Wang received his Ph.D. degree in Engineering Science from Washington State University in 2002. Most recently, he is working in the Department of Biosystems Engineering, and Department of Fisheries & Allied Aquacultures at Auburn University as an associate professor. His research group focuses on applied and fundamental study of food engineering and food safety along with some work on bioenergy.