

## **ANALYSIS OF INTERACTIONS OF PROTEASE INHIBITORS WITH PROTEASES IN A HIGH-THROUGHPUT FORMAT.**

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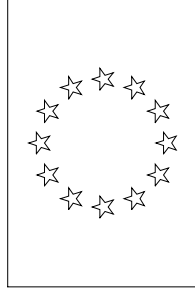
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## ABSTRACT 6

A collection of protease inhibitors was screened for the interaction with various serine (trypsin, cathepsin B) and cysteine (papain, ficin, bromelain) proteases possessing cleavage activity towards a Dabcyl pentapeptide fluorogenic substrate. Protease inhibitor's instability (constant of protease-inhibitor complex) was determined prior to their use in the arrayed format. The inhibitors used were: At-CYST, Arabidopsis thaliana cysteine protease inhibitor; MTI2, mustard trypsin inhibitor, ( $K_i=0.01$  nM) MTI-2-AAA mutant without inhibitory activity; MTI-2-PLI mustard chymotrypsin inhibitor,  $K_i=60$  nM (toward chymotrypsin) and 34 nM toward trypsin; MTI-2-PAI 510 nM (toward trypsin), no activity toward chymotrypsin; Chymo8, chymotrypsin inhibitor,  $K_i=32$  nM (toward chymotrypsin), 161 nM toward trypsin; RTI-2, rapeseed glutamyl inhibitor (no activity toward trypsin and chymotrypsin; active against Streptomyces griseus glutamyl protease); Silk Protease Inhibitor (six variants with one modified amino acid in the P1 site: Thr, Trp, Ala, His, Met, Asp) with various efficiencies in inhibiting chymotrypsin and trypsin; Sb-TI, soybean trypsin inhibitor, from a commercial source. Inhibitors were spotted onto Schleicher-Schuell nitrocellulose covered glass slides, and proteases were labelled with Cyanine-3 before hybridisation with the nitrocellulose-bound inhibitors. Data will be presented on the specificity of the binding and residual activity of proteases.

## INTRODUCTION

Protein chip is a new technology in rapid and constant development.

Proteins can be spotted denatured (silane glass) or in native form (coated glass, hydrogel, nitrocellulose or nylon membrane coated slides). Proteins can be attached via absorption, peptide tags, or by a Schiff base reaction to aldehyde substrates. Protein arrays can be useful tools to: study two interacting proteins, as antibody and antigens; to use arrayed peptide substrates to detect kinase activities and protein phosphorylation. To test the feasibility of protein chip analysis, the interaction between proteases and protein inhibitors was chosen as a model. Recently a huge number of plant protease inhibitors have been found in plant genomes. Heibges and Ghebart (Mol Gen Genomics 2003) identified 3 classes of Kunitz-type protease inhibitors with more than 28 genes in potato: the number of genes and their sequence vary between different varieties. Protease inhibitors could be good agricultural biomarkers to analyse different varieties of plant species and at the same time to screen the inhibition specificity of various PIs.

• In this study was analyzed the protease inhibitory (PI) activity of a set of plant and animal protease inhibitors, which were obtained in the form of recombinant wild-type proteins or as mutants possessing an amino acid substitution that affected their protease activity or specificity. In addition, we tested the binding of PIs to nylon coated slides, and the interaction with trypsin and chymotrypsin that were labeled with cyanine 3. Aim of this work was to test the feasibility of proteinase inhibitors in an array format as a tool to discriminate their specificity of inhibition towards selected protease targets and residual protease activity, with the aim to develop an assay for measuring the activity of proteases bound to glass slides in presence of interacting PIs.

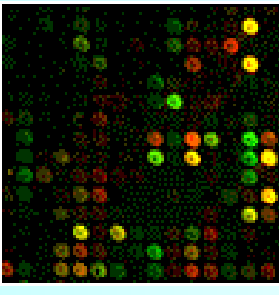


Table 1. Inhibition activity, protease specificity and  $k$  dissociation of purified proteins

At-CYST, *Arabidopsis thaliana* cysteine protease inhibitor;

MTI-2, mustard trypsin inhibitor, ( $K_i=0.01$  nM)

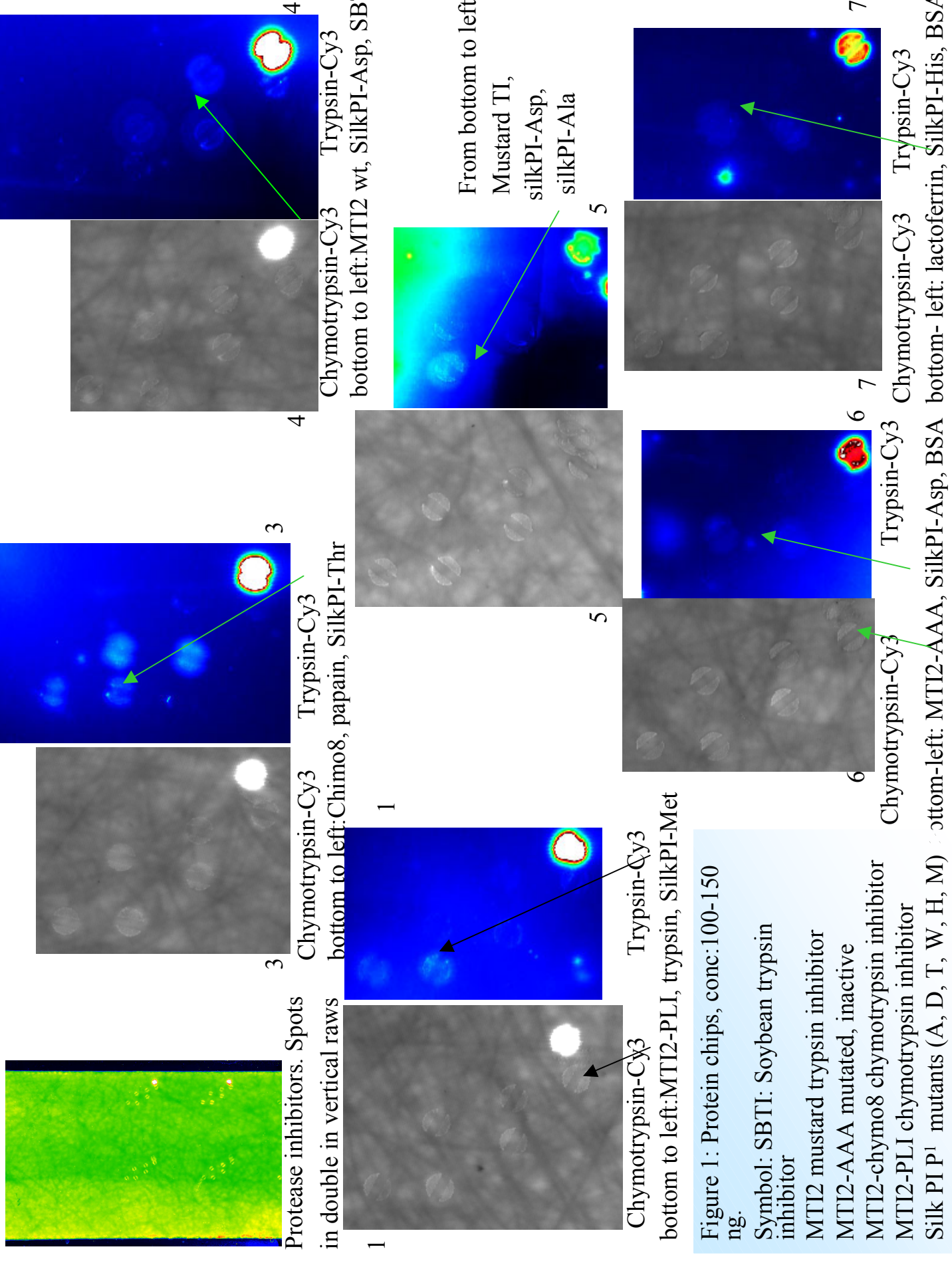
MTI-2-AAA mutant without inhibitory activity;

MTI-2-PLI mustard chymotrypsin inhibitor,  $K_i=60$  nM (toward chymotrypsin) and 34 nM toward trypsin;

MTI-2-PAI, 510 nM (toward trypsin), no activity toward chymotrypsin;

Chymo8, chymotrypsin inhibitor,  $K_i=32$  nM (toward chymotrypsin), 161 nM toward trypsin;

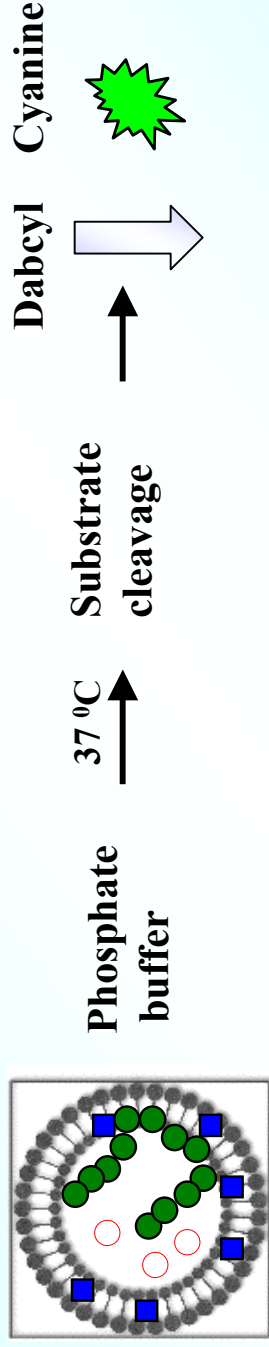
RTI-2, rapeseed glutamyl inhibitor (no activity toward trypsin and chymotrypsin; active against *Streptomyces griseus* glutamyl protease);



Protease catalytic pocket      Inhibitor      Substrate      Enzyme/active Inhibitor/Substrate



Enzyme/inactive Inhib/Substrate



**Figure 2:** A modified assay-on-array protocol was developed with fluorescence-developing substrates and recombinant enzymes. The assay includes: 1) mixing of enzyme with inhibitors prior to reaction initiation (one used as bait, the second as interacting partner), 2) use of Blocking reagent to limit non-specific binding, 3) use of substrate containing a DabcyI- and a cyanine-3 group at the extremity, 4) elimination of the amino acid-bound dabcyI group during proteolytic cleavage and 5) miniaturization of the assay to a final assay volume of 20ul under a cover slide.

## DISCUSSION

A collection of serine protease inhibitors was screened in inhibition assays measured as inhibition of cleavage of benzyl-Arginyl-naphthylamide and benzyl-Phenylalanyl-like substrates, and for the interaction with the proteases trypsin and chymotrypsin.

Most of the protease inhibitors showed high affinity of binding to trypsin or chymotrypsin, as also some of the inactive mutants. Physical interaction with trypsin and chymotrypsin were detected in the protein chip assay for many SPI-mutants: hence binding to protease and accessibility of substrates to the protease active site are two distinct features. Interaction with proteases not necessarily precludes accessibility of catalytic site to small-sized protease substrates.

Steric hindrance, affecting substrate viability to the catalytic pocket and hydrogen bonding amino acids were the major factors involved in inhibition efficiency. Further experiments are ongoing to produce high-density spots and test fluorogenic substrates on a protease chip format.