

Novel Detection Methods Used in Conjunction with Affinity Chromatography for the Identification and Purification of Hydrolytic Enzymes or Enzyme Inhibitors from Insects and Plants

Alexander V. Konarev¹ and Alison Lovegrove²

¹All-Russian Institute for Plant Protection

²Rothamsted Research

¹Russia

²UK

1. Introduction

Plant proteinaceous α -amylase and proteinase inhibitors play many important roles *in planta* as endogenous enzyme regulators and as protective factors against harmful organisms. They are also of interest as antinutrients in human and animal feed, and as anticancer and antiviral agents in medicine. They may also be used as genetic markers in the study of plant diversity and evolution (Dunaevsky et al., 2005; Franco et al., 2002; Gatehouse, 2011; Habib & Fazili, 2007; Konarev, 1996; Konarev et al., 2002b; Mosolov and Valueva, 2005; Shewry & Lucas, 1997). Affinity chromatography is an effective method for the fast purification of various hydrolytic enzymes and their inhibitors. Its use in conjunction with detection methods that can quickly and easily identify components of proteins mixtures with the sought-after activity can appreciably simplify the search and purification of novel enzymes or enzyme inhibitors, especially those with low or non-typical substrate specificities. Advances have been made in fractionation and detection methods by many laboratories but we describe below approaches used by us including the use of affinity chromatography in combination with novel detection methods to identify and purify novel forms of insect and fungal proteinases and also proteinase inhibitors from plants (Connors et al., 2007; Konarev, 1985, 1986, 1990a, 1996; Konarev & Fomicheva, 1991a; Konarev et al., 1999a, 1999b, 2000, 2002a, 2004, 2008; 2011; Luckett et al., 1999). In some cases these have been used for the analysis of the diversity of α -amylase and proteinase inhibitors in various plant taxa (Konarev 1982a, 1986b, 1987b, 1996, Konarev et al., 1999c, 2000, 2002a, 2002b, 2004). The aim of the present chapter is to bring to a larger audience and summarize published works that are hard to access to non-Russian readers or described in scattered publications and to provide some examples using the approaches described. These data have been obtained in the All-Russian Institute for Plant Protection (VIZR) from 1981 and, after 1996, in collaborative work of VIZR with Rothamsted Research, University of Bristol (UK), National Institute of Agrobiological Sciences (Japan) and Hacettepe University (Turkey).

2. Methods for protein fractionation and the identification of enzymes and their inhibitors

2.1 General description of approaches

Affinity chromatography is widely used in the purification of α -amylases, proteinases and the inhibitors of these enzymes in plants, animals and insects (Buonocore et al., 1975; Nagaraj & Pattabiraman, 1985; Saxena et al., 2010). Various methods for the detection of α -amylase inhibitors (Giri & Kachole, 1996; Fontanini et al., 2007) and proteinase inhibitors (Pichare & Kachole, 1994; Mulimani et al., 2002) in protein mixtures are known. We have developed a set of universal and sensitive methods for the detection of inhibitors of various insect, plant, fungal and mammalian α -amylases and proteinases (Konarev 1981, 1982b, 1985, 1996; Konarev and Fomicheva, 1991a; Konarev et al., 1999b, 2000). These are most often used with polyacrylamide gel electrophoresis (PAGE), isoelectric focusing (IEF) and thin layer gel-filtration (TLGF) and in such variants may also be used to search for novel forms of enzyme inhibitors or to monitor the affinity chromatography and other protein fractionation techniques. The distinctive feature of the methods for detection of α -amylase inhibitors we describe is the use of polyacrylamide gels (PAG), containing both amylase and starch. At PAGE protein sample is separated and inhibitors are detected in such gel but at IEF or TLGF this gel serves as 'replica' for detection. In case of proteinase inhibitors sensitive 'gelatin replicas' are used allowing inhibitors for up to 4 different proteinases (or inhibitors of α -amylases and proteinases) to be detected among proteins separated within the same gel.

2.2 Protein samples

Plant seeds were obtained from the collection of the N.I. Vavilov Institute of plant industry, St. Petersburg, (VIR, accessions designated by number "k-.."), the Botanical institute, St. Petersburg, various seed companies (UK) or collected on expeditions by the author. Proteins were isolated from ground seeds or vegetative organs homogenized with different extractants depending upon which proteins were of interest (water, 2 M urea, 7.4 mM Tris - 57 mM glycine buffer pH 8.3 etc.) in the various ratios (w/v) ranging from 1:2 to 1:10. The extractions were carried out at 20° C for 0.5-1 h. For enzymes showing greater sensitivity to inhibitors, the extract was diluted by 50 to 1000 fold. Extracts were heated for 15 min at 80° C for inactivation of endogenous α - and β -amylases and proteinases and denatured proteins were removed by centrifugation. The sources of α -amylases and proteinases for analyses were fresh or freeze dried salivary glands, guts and homogenates of insects, human saliva and wheat grains germinated for 2 days. Commercial preparations (α -amylases from pig pancreas or fungal cultures, trypsin, chymotrypsin, subtilisin, elastase, papain, ficin were from Sigma or other specialist suppliers). α -amylases and proteinases were extracted from homogenized material in water or tris-glycine buffer with or without 1mM CaCl₂. Single bands of amylases or proteinases have been isolated using micropreparative variants of IEF or affinity chromatography and used for detection of inhibitors. In most cases the amylases present in total extracts, when used in detection methods, showed clear pictures of interaction with their inhibitors comparable with those obtained with enzymes extracted from guts or partially purified.

2.3 Methods for protein fractionation

2.3.1 Flat-bed-PAGE in homogenous buffer system

This type of electrophoresis was chosen since it allowed the inclusion of α -amylase in the gel without effecting its concentrating during the separation of proteins which is in contrast to the discontinuous buffer system (see in 2.4.2). The solution containing 6% acrylamide, 0.26 % N,N'-methylenebisacrylamide, 7.4 mM Tris - 57 mM glycine buffer pH 8.3, riboflavin and TEMED was poured into a casting form consisting of two glass plates, one of which possessed juts for forming slots for samples. The 2 mm thick gel was polymerized in the light. The thickness of the gel could be reduced if a plastic supporting film Gelfix for PAGE (Serva) was used providing a covalent binding to the gel layer. The length of the gel was in the range 10 to 20 cm and the position of the slots was determined depending on analysis. The gel was put on the cooling plate of the Multiphor II Electrophoresis unit (LKB) and connected to electrode buffer tanks by paper wicks. Electrode buffers were the same as in the gel. Protein samples were loaded in slots in a volume of between 0.5-20 μ l. Wheat seed albumins were separated in 120x200 mm gel with a path length for bromphenol blue of 9 cm for 1 h at voltage 1000 V, current 40 mA and 10° C. Wheat protein bands were visualized by transfer of the gel into 10 % TCA.

2.3.2 SDS-PAGE and mass-spectrometry (MS)

SDS-PAGE of proteins was carried out according to Laemmli (1970) with modifications. Proteins were heated in a standard SDS-PAGE sample buffer with 2-mercaptoethanol (for estimation of sizes of peptides) or without reductant (for following detection of intact inhibitors). For Edman sequencing proteins were transferred to PVDF membrane using a semi-dry method and CAPS buffer.

For MS peptides were isolated from PAGE and digested by trypsin. Peptide digests were mixed 1:1 with matrix (2 mg/mL of R-cyano-4-hydroxycinnamic acid was dissolved in 49.5% (v/v) ethanol, 49.5% (v/v) acetonitrile, and 1% (v/v) of a 0.1% (v/v) TFA solution). 1 μ l of the mixture was spotted onto the target plate and the sizes of peptides were determined using a Micromass M@LDI-LR mass spectrometer (Waters, Manchester, UK) using a standard peptide mass fingerprinting method and mass acquisition between 800 and 3500 m/z. The laser firing rate was 5Hz, 40 random aims per spot, 10 shots per spectrum, 10 spectra per scan, 10 scans combined, 10% adaptive background subtracted, smoothed (Savitzky-Golay) and centroided. The MALDI-MS was tuned to 10,000 FWHM (Full Width at Half Height) and calibrated with a tryptic digest of alcohol dehydrogenase following the manufacturer's instructions.

For sequencing by MS peptides were concentrated and desalted using Zip-Tips (Millipore), dried, dissolved in 70% (v/v) methanol containing 1% (v/v) formic acid, sonicated for 3 min and then loaded into nanoflow tips (Waters). Electrospray ionization-MS was performed on a quadrupole time-of-flight (Q-TOF) I mass spectrometer (Micromass, Manchester, UK) equipped with a z-spray ion source. Instrument operation, data acquisition and analysis were performed using MassLynx/Biolynx 4.0 software. The sample cone voltage and collision energy were optimized for each sample. The Micro channel plate (MCP) detector voltage was set at 2800 V. Scanning was performed from m/z 100-3500. Prior to acquisition

the mass spectrometer was calibrated using a solution of [Glu¹]-fibrinopeptide B as described by the manufacturer.

2.3.3 Isoelectric focusing (IEF)

IEF of proteins was carried out on a variety of available IEF gel systems (Servalyt Precotes pH 3-10 (Serva) etc.) using LKB Multiphor II apparatus or a Phast System (Pharmacia). Cytochrome c (pI 10.65), horse myoglobin (pI 7.3) and whale myoglobin (pI 8.3) (Serva) were used as markers. Proteins were detected in gels by Coomassie R-250 in 10 % TCA after removal of ampholites by washing in 10 % TCA alone.

For micropreparative IEF, 0.5–1 mg of proteins (e.g. trypsin or chymotrypsin inhibitors eluted from affinity columns) were applied in 0.4 ml water onto a 10x60-mm filter-paper strip across the pH gradient of a Servalyt Precotes (Serva) gel, and IEF was carried out using a Multiphor with a distance of 10 cm between electrodes. The gel was then placed in 40% (w/v) ammonium sulphate for 5 min and the opalescent protein bands excised and placed in 0.5 ml of water for 1 h to elute the inhibitors. Micropreparative IEF in PAG or in Ultradex was used also for the isolation of insect and fungal α -amylases and proteinases.

Micropreparative fractionation of proteinases or inhibitors with higher resolution included IEF in DryStrips (GE Healthcare). Protein fractions obtained by affinity chromatography (e.g. proteinase inhibitors from *Cycas siamensis* or *Veronica hederifolia* seeds) were loaded onto two Immobiline DryStrip pH 3–10 NL gels and inhibitor bands located using gelatin replicas of one gel. The second gel was fixed in 10% (v/v) TCA revealing proteins as opaque bands. Zones containing protein were excised and TCA was removed by washing with cold acetone. Proteins were then extracted from the gel with 20% (v/v) ethanol containing 0.1% (v/v) TFA, followed by 30% (v/v) acetonitrile in 0.03 M ammonium bicarbonate and 4% (v/v) formic acid (for 1 h each) and finally freeze dried. Transfer of proteins from the electrophoresis, IEF or TLGF gels to nitrocellulose (NC) or PVDF membranes was performed using equipment for electro transfer or simple diffusion by contact of NC with gel for 1-12 hours (if detection of inhibitors or microsequencing was to be carried out).

2.3.4 Gel-filtration

Preparative gel-filtration of proteins was carried out in 40-100x1.5 cm columns filled with Sephadex G-100 or G-50 (Fine) or in Ultrogel AcA 54 (LKB) with either 0.1 M phosphate buffer pH 7.4 containing 0.2 M NaCl or 0.1 M ammonium acetate pH 7 (the latter if proteins were to be freeze-dried).

Thin layer gel-filtration (TLGF) of plant proteins was performed in 0.4-1 mm layer of Sephadex G-50 or G-100 Superfine (LKB) attached to 250x125 mm a glass plate or to Gel-bond to agarose film (Serva) with 0.1 M NaCl or 0.03 M ammonium acetate as the liquid phase. The Sephadex gel plate was set in apparatus for horizontal electrophoresis and connected to upper and lower tanks containing the liquid phase by filter-paper wicks. Proteins (0.5-10 mcg/ml) were applied on gel in a minimal volume (0.5-2 mcl). Coloured proteins cytochrome c (12.5 kDa), horse myoglobin (17.8 kDa) and bovine hemoglobin (64.5 kDa) were used as markers. The elution rate was adjusted by changing the angle of inclination of the plate (for the spot of cytochrome c it was about 2 cm/h). The fractionation of wheat seed albumins with MW 12-60 kDa lasted about 3 h.

2.3.5 Affinity chromatography of proteinases and proteinase inhibitors

Affinity matrices using proteinases (trypsin, chymotrypsin and subtilisin) or proteinase inhibitors immobilized on CNBr-activated Sepharose or acrylamide or agarose gels were used for purification of proteinase inhibitors from plants and proteinases from insects (Connors et al., 2007; Konarev 1986a, Konarev et al., 2000, 2002a, 2004, 2008, 2009, 2011). For analytical work, certain types of inhibitor or proteinase were pre-absorbed from protein mixtures by modification of the method of Hejgaard et al. (1981) to facilitate the identification of other types of inhibitors or proteinases. 6 ml of a 50% suspension of affinity gel in water (v/v) was added to 30 ml of protein mixture extracted from seeds with water (1:4) and mixed frequently during 30 min period. The affinity gel was removed by centrifugation.

In preparative work with inhibitors highly specific to certain proteinase (for e.g., trypsin inhibitors from wheat grain and immobilized trypsin), 5-30 ml of solution containing inhibitors was passed through a column with 0.5-3 ml of the affinity gel with immobilized proteinase. For higher volume sample (0, 5 - 1 L) 5-10 ml of suspension of affinity gel was added to the solution and after 15-30 min of shaking the gel was collected using a sintered glass filter. The gel was washed with 10-50 volumes of the same solvent as used in the sample for loading (water, 0.2 M NaCl, 2 M urea, 0.1 M ammonium acetate etc.) and then with 10-50 volumes of 0.1 M NaH₂PO₄ with 0.5 M NaCl pH 5.0 and finally with water. The gel was transferred to a column and inhibitors were eluted with 0.015 M HCl. The process was monitored at 280 nm using standard equipment. Fractions obtained were freeze dried and analyzed for the presence of proteinase inhibitors using IEF combined with the gelatin replicas method (see below). Selected fractions were then separated by reverse phase HPLC with a C18 RP Phenomenex column and a gradient of 15-45% (v/v) acetonitrile in 0.1% trifluoroacetic acid.

Similar procedures were used for isolation of proteinases, e.g. grain borer *Rhyzopertha dominica* (Fab.) gut serine proteinases on soybean TI linked to agarose (Sigma) (Konarev et Fomicheva, 1991a; Konarev, 1992b).

In instances where the interaction between proteinase and inhibitor was weak (for e.g. the gluten-specific proteinase of sunn pest *Eurygaster integriceps* Put. and chymotrypsin inhibitor I from potato, Calbiochem), after loading of the sample the affinity gel was washed just with the solvent used in the sample (0.01 M ethanolamine with 0.2 M NaCl and 0.01% Triton X-100 pH 10) and eluted with water/0.01% (v/v) Triton X-100 followed by 0.01M HCl/0.01% (v/v) Triton X-100 (Konarev et al., 2011). Both eluates contained almost pure proteinase.

2.4 Detection of α -amylases and their inhibitors

2.4.1 Detection of α -amylases after PAGE or IEF

For the estimation of heterogeneity and electrophoretic mobility of the α -amylases 0.1 % soluble starch was added to the solution, described in 2.3.1, before polymerization of the gel for PAGE. After separation the gel was placed in 0.1 M acetic pH 5.4 (for insect and germinating wheat grain α -amylases) or 0.1 M phosphate buffer pH 7.0 (for human and mammalian α -amylases) containing 0.1 M NaCl and 1 mM CaCl₂ and incubated for 30 min at 37° C. Areas of α -amylase activity were observed as transparent bands on dark blue

background after immersion of the gel in I/KI solution (50 mg I and 1000 mg KI per liter of water). In case of IEF the gel with the same composition was placed on top of a separating gel and after incubation at 37° C for 10-30 min put in I/KI solution.

2.4.2 Detection of α -amylase inhibitors after PAGE, IEF or TLGF of plant proteins

In order to detect α -amylase inhibitors in complex seed protein extracts 0.1 % starch and the α -amylase of interest were included in separating gel for PAGE (see 2.3.1) before polymerisation. The volume of added α -amylase solution approximately corresponded to the enzyme activity required to hydrolyze all the starch in the gel after 1 h at optimal pH and at 37° C (which was determined in preliminary tests using a row of small gels containing different volumes of α -amylase). The optimal volume of seed protein sample loaded in a slot of the gel depended strongly on the type of α -amylase included in the gel and its sensitivity to certain type of inhibitors. Therefore, when inhibitors of low sensitive α -amylase from sunn pest *E. integriceps* gut were to be identified, 10-15 ml of protein samples extracted from bread wheat seeds (with water or 2 M urea (1:4)) were loaded. In the case of the much more inhibitor-sensitive α -amylase from mealworm beetle *Tenebrio molitor* L. larvae the extract was diluted 500-1000 times before loading. The key features of this method are that (1) the enzyme and substrate are present in the separating gel simultaneously and untimely hydrolysis is prevented by non-optimal pH and a low temperature during separation, (2) α -amylase included in the gel, in most cases, moves slower than the inhibitors and after PAGE in homogenous buffer system inhibitors are evenly surrounded by enzyme and finally (3) the presence of an α -amylase in all layers of the gel (in contrast to methods, where the gel is incubated in a α -amylase solution) provides a very high sensitivity of detection.

In the described conditions of electrophoresis α -amylases from human saliva, pig pancreas, gut of sunn pest *E. integriceps* and germinating wheat seed had a mobility lower than that of wheat albumins including amylase inhibitors. In case of α -amylase of mealworm beetle *T. molitor* larvae the enzyme moves faster than the inhibitors, so that separating gel should be extended in part between sample slots and cathode in order to compensate for the difference in speed of run of enzyme and inhibitors. After separation gels were put in 0.1 M acetic pH 5.4 (for insect and wheat germinating grain α -amylases) or 0.1 M phosphate buffer pH 7.0 (for human and mammalian α -amylases) containing 0.1 M NaCl and 1 mM CaCl₂ and incubated for 30-50 min at 37° C. For initial runs (with an "unknown" α -amylase) hydrolysis of starch should be checked every 5-10 min on small pieces of gel cut from the sides of the separating gel and immersed in iodine solution. Incubation may be stopped when the gel pieces have acquired a violet-pink colour. The whole separating gel can then be transferred to the iodine solution. Inhibitors become visible as dark blue bands of undigested starch on the light transparent background.

For detection of α -amylase inhibitors after IEF or TLGF of plant proteins the gel with the same composition as was used for detection at PAGE (see above) was placed onto separating gel for 10-30 min and then put in buffer with pH optimal for α -amylase and incubated for 15-40 min at 37° C until test pieces of the gel have acquired a violet-pink colour. More details will be given at description of examples of method application.

2.5 Detection of proteinases and proteinase inhibitors

2.5.1 Detection of proteinases

Proteinases were detected in protein mixtures isolated from insect, fungal or plant samples following separation by IEF or PAGE using plastic films covered by protein substrates (gelatin or glutenin) or solutions of synthetic substrates.

The most sensitive and applicable to detection of many serine and cysteine proteinases with wide substrate specificity (trypsin-, chymotrypsin-, subtilisin-, papain- or ficin-like enzymes) method is based on the use of layer of gelatin present in photographic materials. This approach exists in many variants (Burger and Schroeder, 1976; Harsulkar et al., 1998). We used undeveloped opaque photographic film ("Photo 65", Russia, for example) both for analytical and micropreparative scales (Konarev and Fomicheva, 1991a; Konarev et al., 1999b). E.g., for isolation of extracellular proteinases of fungi *Sclerotinia sclerotiorum* and *Colletotrichum lindemuthianum* cultural filtrate was applied to the gel using wide filter-paper strips. After IEF three 1x40 mm strips were placed on the gel along the pH gradient and incubated for 5 min at 40° C. The zones of the gel corresponding to hydrolyzed gelatin were cut out, immersed in 200 ml of 25 % sucrose for 1 h and the solution was used for detection of proteinase inhibitors using the gelatin-replicas method (see below) (Konarev et al., 1999b).

For the detection of glutenin-specific proteinase from the salivary glands of sunn pest *E. integriceps*, a layer of insoluble in acetic acid glutenin was attached to the plastic film as substrate (Konarev et al, 2011).

2.5.2 Detection of proteinase inhibitors

There are many methods for detection of proteinase inhibitors in protein mixtures following separation of the proteins by PAGE or IEF. Some are based on the use of a gelatin layer on photographic film as a substrate for proteinases (Pichare & Kachole, 1994; Mulimani et al., 2002). However, most of these methods include immersion of the separating gel in a proteinase solution followed by laying of photographic film on the gel and incubation. But because of diffusion of proteins from the gel in solution the sharpness of the bands can be decreased. Also, immersion in certain proteinase solutions prevents the detection of the inhibitors of other proteinases in the same gel. We developed the so-called "gelatin replicas" method which includes consecutive contact of the separating gel with one to four replicas which can be developed by four different proteinases. The method is based on ability of gelatin layer to absorb proteins from the separating gel (as with protein blotting onto nitrocellulose) (Konarev, 1986a; Konarev et al., 2002a, 2002b, 2004, 2008). This approach gives also the opportunity to detect α -amylase and proteinase inhibitors in the same separating gel (Konarev, 1986b, 1996).

One to four pieces of undeveloped non-transparent photographic film were applied sequentially to IEF gel (for 2, 5, 20 and 30 min, respectively). The "gelatin replicas" containing inhibitors absorbed from the IEF gel were then applied to 0.8% (w/v) agarose gels containing 0.1 M Na₂HPO₄ (pH 9) and one of serine proteinases (Sigma): trypsin (1 mcg/ml), chymotrypsin (10 mcg/ml), elastase (4 mcg/ml) and subtilisin (0.3 mcg/ml), or some insect or fungal serine proteinase. Incubation for 30 min at 45° C allowed the proteases to digest the gelatin on the photographic film with the positions of inhibitors being detected

as dark "islands" of undigested gelatin on the photographic layer. The similar approach was used for detection of plant and insect cysteine proteinase inhibitors (Konarev, 1984; 1986a,1990a; Konarev et al, 1999a, 2002b), 0.1 M NaH₂PO₄ with 0.1 M DTT being included in agarose gels together with proteinase.

Besides, gelatin films could be effectively used for detection of inhibitors on NC replica from separating gel, soaked with proteinase solution and washed by buffer. This approach can be used, for example, for detection of inhibitors after SDS-PAGE or in "cross" method for improving of resolution of inhibitor or enzyme spectra.

2.5.3 Cross methods for detection of inhibitors of single α -amylase or proteinase IEF bands

In order to identify inhibitors of individual proteinase bands in complex proteinase mixtures without special purification of proteinases or inhibitors, the "cross" methods has been developed (Konarev, 1990a; Konarev et Fomicheva, 1991a). Proteinases and protein mixture containing inhibitors extracted from seeds were applied to the individual gels using wide paper strips (about 9 cm for 120x120 mm gel). At low contention in extract, inhibitors could be preliminary enriched by affinity chromatography. After separation a photographic film was placed for 20 min on the gel to obtain a replica of fractionated proteins and then a gelatin replica was applied to the gel with separated proteinases, at right angles to each other, and incubated for 0.5-1 h at 38° C. Narrowings or brakes in the proteinase bands corresponds to positions of their inhibitor bands. In our hands this method is applicable for the inhibitors from mature wheat grain and serine and cysteine proteinases from insects and germinating grains. The same approach was used for α -amylases and their inhibitors (see 3.1.).

3. The use of detection methods and affinity chromatography in study of hydrolases and their inhibitors in relation to the problems of plant diversity, evolution, pest resistance, food quality and medicine

3.1 Analysis of polymorphism of α -amylase and proteinase inhibitors in plants

The detection methods may be used for studying plant diversity and evolution by analysis of polymorphism of α -amylase and proteinase inhibitors. They may also find utility in the field of pest resistance and food quality. The figures that follow demonstrate some examples of application of the methods described.

It is well known that exogenous α -amylase inhibitors in wheat and related cereals are represented by three main fractions: 12 kDa (with monomeric molecules), 24 kDa (dimeric) and about 60-66 kDa (tetrameric) which can be easily obtained by gel-filtration (Buonocore et al., 1977; Franco et al., 2002). Fig. 1, I, shows results of PAGE of different fractions of wheat grain proteins followed by detection of inhibitors of insect, mammalian and plant amylases. α -amylases differ in sensitivity to various fractions of inhibitors, α -amylase from pig pancreas being intermediate between α -amylase from human saliva and insect amylases in their interaction with monomeric inhibitors. The most of studied insect α -amylases were inhibited by 12 kDa albumin fraction in contrast to human α -amylase. α -amylase from germinating wheat grain (c) and exogenous α -amylases are inhibited by different protein components. The method allows detection of inhibitors of both highly-sensitive (to

inhibitors) and low-sensitive α -amylases. So, for obtaining comparable visible inhibitor bands, in case of highly sensitive α -amylase from *T. molitor* larvae, amount of seed proteins applied to PAGE was approximately in 1,000 times lower than that for α -amylase from sunn pest *E. integriceps* gut. With the same amount of seed proteins as was used for gut enzyme, α -amylase from sunn pest salivary glands was not inhibited at all (not shown) that indicated its practically full insensitivity. The reason for the variation in sensitivity of mentioned α -amylases to inhibitors may be hypothesized to be the co-evolution of the insects and plants on the level of digestive enzymes and their inhibitors. For example, sunn pest is a highly specialized phytophage (in contrast to *T. molitor*); during co-evolution of this bug with wheat its digestive amylases developed decreased sensitivity to inhibitors from wheat grains that weakened negative role of this proteins (Konarev, 1981, 1996). The same can be true for the sunn pest digestive proteinases, insensitive to inhibitors from wheat grain (Konarev et al., 2011; Konarev and Fomicheva, 1991a) and also for proteinases of some other insect pests (Gatehouse, 2011).

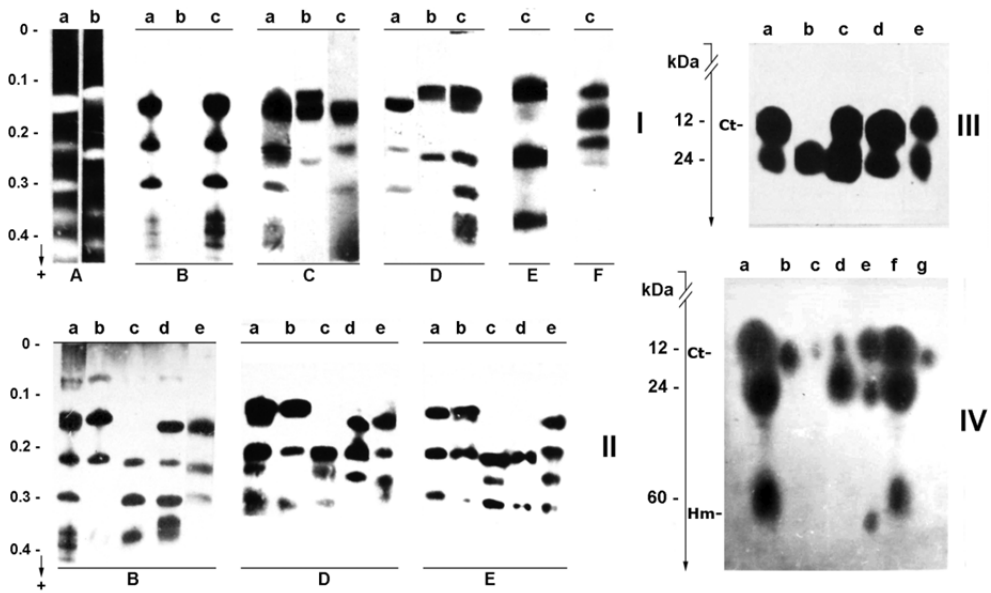
Bread wheat is a hexaploid (amphidiploid) plant combining three different genomes that determines the specific composition of wheat proteins (V.G. Konarev, 1996; Shewry et al., 2003) including enzyme inhibitors. Fig. 1, II, shows the variability of inhibitors of three α -amylases in grains of hexaploid bread wheat *Triticum aestivum* (genome composition A^aA^bBBDD), tetraploid *T. turgidum* (A^aA^bBB) and diploid goatgrass (*Aegilops*) species related to donors of genomes D and B for *T. aestivum*.

Methods of detection can be efficiently used in screening of plant collections for inhibitor composition. Presence or absence of main α -amylase inhibitor fractions in accessions can be easily estimated using thin layer gel filtration (TLGF) in combination with a PAG replica, containing starch and target enzyme (Fig1, III&IV). So, accessions of *Ae. speltooides* from the World collection of Vavilov Institute of Plant Industry (III, b & c) differ in presence of monomeric inhibitor of insect α -amylase. Fig.1, IV shows main insect amylase inhibitor fractions present in wheat, wild and cultivated barley species, maize, and oat grains (Konarev & Fomicheva, 1991b; Konarev, 1992b).

Using of TLGF with detection methods appeared to be also effective in search for novel low-molecular weight proteinase inhibitors (see in 3.2.2 and 3.2.3).

IEF in combination with PAG-amylase-starch replica provided much higher resolution of protein fractionation (Fig.2) although with less sensitivity of inhibitor detection.

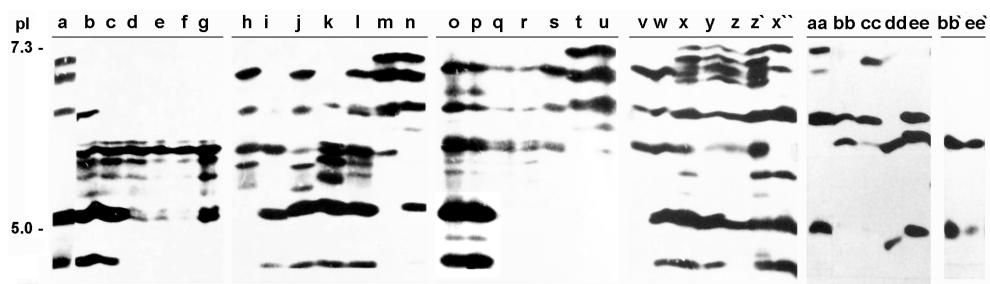
This approach was most suitable for work with inhibitors (from cereals) of highly sensitive α -amylase of *T. molitor* larvae, and was also applicable to inhibitors of α -amylases from beetles lesser grain borer *Rhyzopertha dominica*, granary weevil (*Sitophilus granarius* L.) and human salivary α -amylase, but was not effective enough for sunn pest *E. integriceps* gut α -amylase inhibitors. These methods were used for analysis of hundreds of accessions of wheat and related cereals for α -amylase inhibitor composition in relation to problem of wheat diversity, evolution and pest resistance (Konarev, 1982a, 1986b, 1992b), and also for analysis of variability of Mexican bean weevil (*Zabrotes subfasciatus*) and azuki and bean weevil (*Callosobruchus chinensis*) α -amylase inhibitors among seed proteins of *Phaseolus* and *Vigna* accessions (Konarev et al., 1999c). Clear evolutionary links between the α -amylase inhibitor systems in bread wheat and in other wheat and *Aegilops* species related to genome donors to *T. aestivum* were established during researches with use of mentioned method.



Panels I & II. 6% PAG containing 7.4 mM Tris - 57 mM glycine buffer pH 8.3 and 2 M urea (A), and additionally 0.1 % starch and α -amylases from: human saliva (B), pig pancreas (C), sunn pest (*Eurygaster integriceps* Put.) gut (D), mealworm beetle larvae (*Tenebrio molitor* L., E) and germinated wheat grain (F). I, panels A-F contain tracks of separated wheat proteins: a & b, bread wheat (*Triticum aestivum*) seed albumins 12 kDa (a) and 24 kDa (b), and total proteins extracted from wheat flour with 2 M urea (c). Panel II, B-E: tracks a-e, seed proteins extracted with tris-glycine buffer; a - *T. aestivum*; b, *Aegilops tauschii*; c, *Ae. longissima*; d, *T. turgidum*; e, *Ae. speltoides*. Proteins in separating gel A (I), were detected by fixation in 10 % TCA. α -amylase inhibitors were detected in separating gels (I, A-F and II, B, D & E) as described in 2.4.2. Panels III & IV. TLGF of seed proteins extracted by tris-glycine buffer in 0.4 mm layer of Sephadex G-100 Superfine. Ct & Hm, positions of coloured marker proteins cytochrome c and hemoglobin (spots not shown). 12, 24 and 60, approximate MW of inhibitor fractions. Panel III: a, *T. turgidum*; b & c, *Ae. speltoides* k-443 & k-1596; d, *Ae. longissima* k-194; e, *Ae. bicornis* k-904. Panel IV: a & f, *T. aestivum*; b & g, *Zea mays*; c, *Avena sativa*; d, *Hordeum vulgare*; e, *H. bulbosum*. The 6% PAG-replica containing 0.1 % starch, *T. molitor* α -amylase and tris-HCl buffer pH 8.3 has been placed on separating gel for 15 min and α -amylase inhibitors were detected (see in 2.4.2).

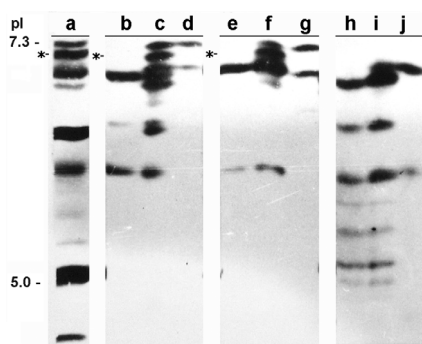
Fig. 1. Detection of α -amylase inhibitors from wheat and other cereals seed following either PAGE (I & II) or thin layer gel-filtration (TLGF, III & IV).

Fig. 2. demonstrates one of the fragments of analysis of the variability of insect (*T. molitor*) and human saliva α -amylase inhibitors (tracks bb' & ee') in accessions of cultivated and wild wheat, *Aegilops* and rye species. The use of mentioned detection methods allowed us to find first that monomeric insect α -amylase inhibitors are controlled by chromosomes 6B and 6Da of wheat (Konarev, 1982 & 1996) and estimate the level of inter- and intraspecific variation of wheat by insect inhibitor composition and activity which impacts on the nature of pest resistance of wheat. Short reviews of these results on α -amylase inhibitors were published in English (Konarev, 1996, 1999a, 2000). Here we describe just examples of application of detection methods.



a & n, *Aegilops speltoides* k-1596; b, *Triticum militinae*; c & k, *T. timopheevii* k-29553; d, e & g, *T. araraticum*, different accessions; f, *T. zhukovskiyi*; h, s & v, *T. persicum*; i, *Ae. longissima* k-194; j & w, *T. turgidum* k-42267; l, o & p, *T. timonovum*; m, t & u, *Ae. speltoides* k-452, k-443 & k-1316; q & r, *T. fungicidum*; x, y, z & z', *T. aestivum* (x & y, var. Bezostaya 1; z & z', var. Chinese Spring; x'' & aa, *Ae. tauschii*; bb & bb', rye *Secale cereale* var. Vyatka 2; cc, *S. segetale*; dd, *S. montanum*; ee and ee', *S. ancestrale*. Replicas containing 0.1% starch and *Tenebrio molitor* α -amylase (a-g, h-n, o-u, v-x'' & aa-ee) and human salivary α -amylase (bb'-ee') were placed on separating gels and developed as described in 2.4.2.

Fig. 2. Detection of α -amylase inhibitors after IEF of wheat and other cereals seed proteins.



IEF of endosperm proteins and detection of inhibitors as described in 2.4.2. a, *Triticum aestivum*; b & h, *T. persicum*; d & g, *Aegilops tauschii* k-80; e & j, *T. boeoticum* (accession No 21); c, mixture of extracts b & d; f, mixture of extracts e & g; i, mixture of extracts h & j.

Fig. 3. Analysis of formation of hybrid molecules ("*") of dimeric *Tenebrio molitor* α -amylase inhibitors (24 kDa) *in vitro*.

The inhibitor set in hexaploid *T. aestivum* (genome formula A^uA^uBBDD) combines sets of inhibitors from *T. turgidum* (A^uA^uBB) and *Ae. tauschii* (DD), but additional components are visible (Fig.2 & 3). Fig 3, a-d, indicates that these inhibitor bands correspond to novel bands emerging in the mixture of proteins from *T. persicum* accession (species possessing the same genome composition as *T. turgidum*, but deprived of monomeric α -amylase inhibitors) and *Ae. tauschii* accession k-80 (rare form deprived of monomeric inhibitors). Presumably novel bands have hybrid nature and arose as a result of interchange of subunits of dimeric inhibitors controlled by different genomes. Another model mixture of proteins of *Ae. tauschii*

(DD) and diploid *T. boeoticum* (A^bA^b , rare accession possessing dimeric amylase inhibitor) also gave hybrid bands (f) in contrast to mixture of *T. persicum* and *T. boeoticum* proteins (i). Results indicate that hybrid bands emerge in combination of subunits controlled by B and D genomes or A and D genomes, and are absent in combination of subunits controlled by A and B genomes, that can be used as criteria for estimation of affinity of subunits.

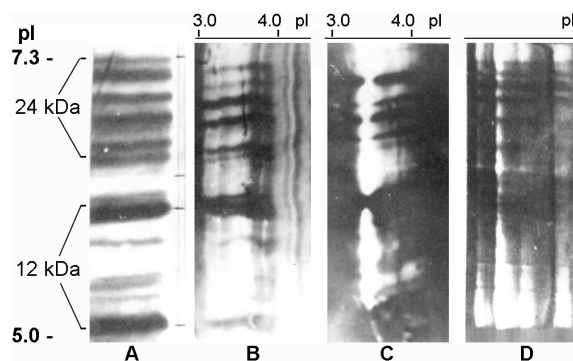
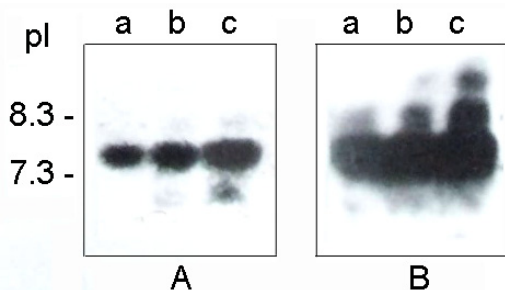


Fig. 4. Detection of inhibitors of α -amylase components using "cross"-method.

Protein extracts containing inhibitors and α -amylases were loaded onto detached gels with wide filter paper strips and separated by IEF. PAG replicas B-D containing 0.4 % starch were placed first onto the gel with separated inhibitors. After 15 min they were transferred onto the gel with separated α -amylases with a rotation of 90° relatively to the initial direction of separation. After a further 15 min, replicas were placed in a buffer with pI optimal for α -amylase and finally after 20 min moved to an iodine solution for staining. Replica from gel A contained *Tenebrio molitor* α -amylase and 0.4% starch and was used to demonstrate the spectrum of insect amylase inhibitors. Vertical direction on figure: IEF of water-soluble proteins from bread wheat var. Bezostaya 1 endosperm. Extracts were loaded on the gel using wide filter paper strips (A, B, C and D, loaded 2, 20, 20 and 10 mcg per mm of strip respectively). Horizontal direction: IEF of α -amylases from lesser grain borer (*Rhyzopertha dominica* F.) adults (B), granary weevil (*Sitophilus granarius* L.) adults (C) and human saliva. 12 and 24 kDa - approximate areas of protein spectrum with mainly monomeric and dimeric forms of inhibitors.

The "cross" variant of detection method can be useful for the preliminary characterisation of interaction of enzymes and inhibitors in complex mixtures. Fig. 4 demonstrates the uses of this approach by analyzing the interaction of individual bands from IEF inhibitor spectra with individual bands of heterogeneous α -amylase samples without special purification both of inhibitors and enzymes. In this case all IEF bands of each separated amylase are inhibited by similar inhibitor bands that indicate homogeneity of revealed enzyme bands in each analysed sample by sensitivity to inhibitors. The same approach was used for various serine and cysteine proteinases from insects and plants and their inhibitors (see method description in part 2.5.3). It allowed to reveal differences of bands of certain IEF proteinase

spectrum in sensitivity, for example, to trypsin or chymotrypsin inhibitor bands and, correspondingly, to classify proteinase bands. In case of low concentration, inhibitors can be preliminary enriched by affinity chromatography (Konarev, 1990a; Konarev and Fomicheva, 1991a).



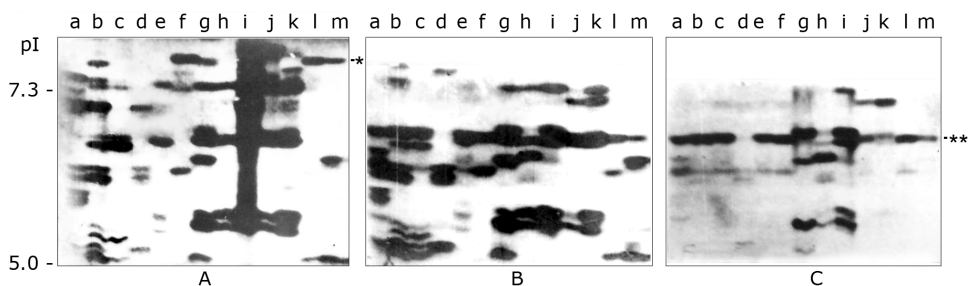
a, b c, 1, 3 & 4 mcl of protein solution extracted from *Hordeum bulbosum* endosperm with water (w/v 1:4) were loaded and separated by IEF. A PAG replica containing 0.4 % starch and *Tenebrio molitor* α -amylase (A) and gelatin replica (B) were put on the same gel sequentially and developed for either amylase or trypsin inhibitory activity.

Fig. 5. Detection of bifunctional α -amylase/trypsin inhibitor in protein spectra.

PAG-amylase-starch and gelatin replicas can be used in parallel for the detection of bifunctional α -amylase/proteinase inhibitors. Fig. 5 shows the results of such an approach for the revealing of the bifunctional insect α -amylase/trypsin inhibitor in IEF spectra of proteins from grains of wild barley species *Hordeum bulbosum* (Konarev & Fomicheva, 1991b; Konarev, 1992b). Bifunctional α -amylase from germinating wheat grain/subtilisin inhibitor and insect α -amylase/trypsin inhibitor have been detected in wheat and maize grains by the same methods (Konarev 1985, 1986b, 1992b). Revealing bifunctional nature of certain inhibitors opens up wide opportunities for their purification by affinity chromatography.

Sometimes the composition of inhibitors in the sample appears to be very complicated but the using of a combination of the gelatin replica method with a simplified version of affinity chromatography, affinity adsorption, can be useful. IEF spectra of proteins isolated from wheat and related cereals grains contain chymotrypsin, subtilisin (Fig.6., A&B), trypsin and amylase inhibitors (replicas not shown). The gelatin replicas A & B were obtained from the same separating gel sequentially and developed by chymotrypsin and subtilisin respectively. This showed that most of the bands revealed in replicas A and B coincided and corresponded to chymotrypsin/subtilisin inhibitors. Other bands have been revealed just in one of the replicas. A third replica from the same IEF gel developed by trypsin (not shown) allowed us to identify trypsin/chymotrypsin inhibitors (indicated on replica A with “*”) in protein spectra of diploid wheat species *T. monococcum* (f), *T. boeoticum* (l) and *T. urartu* (m) and also in one of two accessions of *Ae. longissima* (g). In order to identify more clearly subtilisin inhibitors, the chymotrypsin/subtilisin inhibitors were removed from the protein extract by selective affinity adsorption using Sepharose gel with immobilized chymotrypsin. Treated extracts were separated by IEF and a gelatin replica from the gel was developed by subtilisin (replica C). The use of PAG-amylase-starch replica from the same gel

showed that major bands visible on replica C correspond to germinating wheat grain α -amylase/subtilisin inhibitor (**). Most of the analyzed accessions of diploid, tetraploid and hexaploid wheat species and *Ae. tauschii* have similar major band of this inhibitor (except one of the accessions of *T. dicoccoides* in which it is absent). In their turn such inhibitors in accessions of *Aegilops* species from section *Sitopsis* differ from each other and from wheat inhibitors by pI.



Proteins were extracted with water and separated by IEF in pI range 5 to 9 and gelatin replicas A & B were obtained from the same gel sequentially. Chymotrypsin inhibitors were removed from the extracts by affinity adsorption, the remaining proteins were separated by IEF and replica C obtained. Replica A was developed by chymotrypsin and replicas B and C by subtilisin (see in 2.5.2). a, *Ae. tauschii* k-133; b, *T. aestivum* var. Chinese Spring; c, *T. dicoccum* k-81; d, *T. dicoccoides* k-5201; e, *T. durum* var. Novomichurinka; f, *T. monococcum* k-18140; g & h, *Ae. longissima* k-194 and k-178; i- *Ae. bicornis* (k-904); j & k, *Ae. speltooides* k-1316 & k-198; l, *T. boeoticum* k-40117; m, *T. urartu* k-33871. Samples were loaded in volume 20 mcl. *, position of trypsin/chymotrypsin inhibitor; **, position of bifunctional germinating wheat grain α -amylase/subtilisin inhibitor.

Novomichurinka; f, *T. monococcum* k-18140; g & h, *Ae. longissima* k-194 and k-178; i- *Ae. bicornis* (k-904); j & k, *Ae. speltooides* k-1316 & k-198; l, *T. boeoticum* k-40117; m, *T. urartu* k-33871. Samples were loaded in volume 20 mcl. *, position of trypsin/chymotrypsin inhibitor; **, position of bifunctional germinating wheat grain α -amylase/subtilisin inhibitor.

Fig. 6. Analysis of component composition of chymotrypsin and subtilisin inhibitors from endosperm of various wheat (*Triticum*) and goatgrass (*Aegilops*) species.

Bifunctional α -amylase/subtilisin inhibitors from cereal seeds are considered to be involved in fundamental mechanisms providing plant defence and the regulation of endogenous α -amylase activity. So, screening of plant collections using proposed detection methods can assist in the search for novel forms of inhibitor of this type which can be then purified by affinity chromatography.

The gelatin replicas method has been used for studying the proteinase inhibitor polymorphism in seeds and leaves of hundreds of accessions of cultivated and wild wheats and related species. Evolutionary links of proteinase inhibitor systems of diploid and polyploid wheat and *Aegilops* species have been established and the possibility of using of this approach for wheat variety identification was shown (Konarev, 1986b, 1987a, 1987b, 1988, 1989, 1992b, 1993, 1996, 2000). Similar approaches have been used for analysis of changes in inhibitor composition in potato leaves and tubers after mechanical damage or infection of leaves by *Phytophthora infestans* (Konarev & Fasulati, 1996; Konarev, 2000b; Konarev & Zoteeva, 2006).

Use of gelatin and PAG-amylase-starch replicas in combination with IEF allows the study of polymorphism of insect α -amylase and serine and cysteine proteinases inhibitors in some

legumes. The results obtained were generally in agreement with data of other researches on DNA-markers and allowed the taxonomy in section *Ceratotropis* of *vigna* to be clarified (Konarev et al, 1999c, 2002b) and later assisted in the description of novel *vigna* species.

Gelatin replicas combined with IEF, TLGF and affinity chromatography were used for analysis polymorphism and characterisation of serine proteinase inhibitors in seeds of several hundred species of Compositae and other representatives of wide angiosperm group, asterids, which never before were studied for such inhibitors (Konarev et al., 1999a, 2000, 2002a, 2004). The distribution and variability of various inhibitor types have been first studied and novel inhibitor forms have been discovered (see below).

The same combination of methods have been used for revealing and characterisation of serine proteinase inhibitors in seeds of various gymnosperms (Konarev et al., 2008 & 2009).

As addition to methods for detection of α -amylase and proteinase inhibitors after analytical separation in gels, the techniques for identification of other plant proteins with presumably protective role, lectins, have been also developed (Konarev, 1990b).

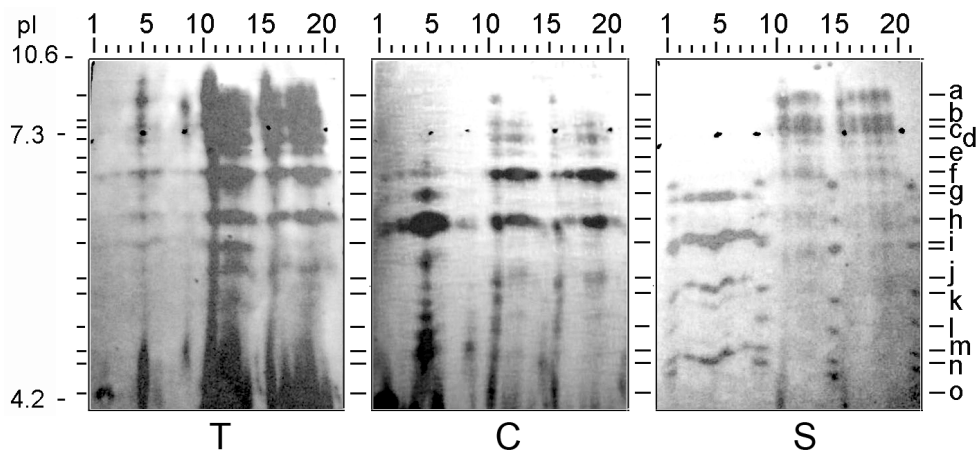
3.2 The search for novel forms of proteinases and proteinase inhibitors and their purification using combination of detection methods and affinity chromatography

3.2.1 Serine proteinase inhibitor from *Cycas* seeds

Proteinase inhibitors (PIs) play an important role in the molecular interaction and co-evolution of plants with phytophagous organisms. Serine PIs have been well studied in angiosperms but until recently not identified in gymnosperms. Among the latter, the Cycadales are of particular interest since they represent the most primitive living seed plants, related to extinct seed ferns, and are sometimes considered a “missing link” between vascular non-seed plants and the more advanced seed plants. With use of gelatin replicas method serine proteinase inhibitors were found in several representatives of two of the four major groups of gymnosperms, the Cycadales and the economically important Coniferales. Inhibitors of subtilisin, a typical enzyme of fungi and bacteria, were identified in members of both orders, being particularly active in the Cycadales. In two *Cycas* species these inhibitors were also active against trypsin and chymotrypsin, proteinases typical of both fungi and animals. Using combination of IEF, gelatin replicas method and affinity chromatography several inhibitor forms from *C. siamensis* seeds have been purified. They appeared to be highly heterogeneous. A small portion of the analytical research, an example of using the gelatin replicas method to monitor the affinity chromatography and determine the degree of heterogeneity and similarity of inhibitor fractions eluted directly from trypsin- and chymotrypsin-Sepharose or consequently eluted from one media after another, is shown in Fig. 7.

For the purification of inhibitor isoform, IEF in DryStrip NL pH 3-10 of fraction obtained by affinity chromatography on chymotrypsin-Sepharose combined with gelatin replicas method was used. Partial sequencing of an isoform showed its similarity to Kunitz-type inhibitors from angiosperms. Analysis of expressed sequence tag (EST) databases confirmed the presence of mRNAs encoding Kunitz-type inhibitors in the Cycadales and Coniferales

and also demonstrated their presence in a third major group of gymnosperms, the Ginkgoales. The results show that gymnosperms and angiosperms contain similar type of serine PIs which may provide protection against microbial pathogens or limit the activity of symbiotic microorganisms (Konarev et al., 2008, 2009).



Proteins were extracted with water and loaded onto affinity chromatography columns, eluted by 0.1 M HCl, and the eluates separated by IEF in Servalyt Precotes pH 3-10. The inhibitors were detected in gelatin replicas by trypsin (T), chymotrypsin (C) and subtilisin (S). 1, 5, 8, 15 and 20, markers pI, position of horse myoglobin (pI 7.3) on replicas marked by ink dots; 2, 8, 14 and 20, proteins extracted from seeds with water; 3-7, eluates from chymotrypsin-Sepharose column; 9-13, eluates from trypsin-Sepharose column on which eluate from chymotrypsin-Sepharose column was loaded; 15-19, eluate from trypsin-Sepharose column. a-o, designations of positions of inhibitor bands.

Fig. 7. Monitoring affinity chromatography and analysis heterogeneity of serine proteinase inhibitors from seeds of *Cycas siamensis*.

3.2.2 Trypsin inhibitor from seeds of veronica with an unusual helix-turn-helix proteinase inhibitory motif

Joint use of IEF, TLGF and gelatin replicas method for screening of seed proteins from numerous representatives of asterids revealed some of them to contain several unusually low-molecular weight trypsin inhibitors. Trypsin inhibitor from ivyleaf speedwell (*Veronica hederifolia* L., Lamiales, Plantaginaceae) seeds (VhTI) has MW near 4 kDa, which is much lower than that of majority of known inhibitors in plants. It was purified using affinity chromatography followed by isoelectric focusing combined with gelatin replica method. The single bands of detected inhibitor were additionally fractionated by reverse phase HPLC (Konarev et al., 2004). At that time no homology with known inhibitors was found. Both the native inhibitor and corresponding especially synthesized peptide have been crystallized and their three-dimensional structures were determined (Connors et al., 2007). It was found that this inhibitor contains an unusual for inhibitors helix-turn-helix proteinase inhibitory motif. The subsequent analysis allowed us to reveal that VhTI is a member of wide group of plant peptides with antimicrobial and inhibitory activities found by other authors (Nolde et al., 2011; Park et al., 1997), first characterized by spatial structure. Their amino acid

sequences can differ greatly from VhTI but also contain four cysteine residues in configuration nX-CXXXC-nX-CXXXC-nX. Further analysis of data clarified that some forms of plant thionins (e.g. α -purothionins) and neurotoxins from scorpions (Chagot et al., 2005) and marine snails (Möller et al., 2005) also possess a similar cysteine configuration and two alpha-helices connected by a loop and stabilized by two disulphide bridges. Today it is clear that effective enzyme inhibitors with some additional features (e.g. antimicrobial activity) could be constructed from simple helical motifs and VhTI and VhTI-like peptides provide a new scaffold on which to base the design of novel serine protease inhibitors e.g. as anticancer drugs for using in medicine.

3.2.3 Unique low-molecular weight cyclic trypsin inhibitor from sunflower seeds

Polymorphism of serine proteinase inhibitors was first studied in sunflower and other Compositae seeds using IEF, TLGF and gelatin replicas methods. Highly active trypsin inhibitors slightly active also to chymotrypsin with pI around 10 and trypsin/subtilisin (TSI) inhibitors with lower pI were detected in seeds of sunflower (*Helianthus annuus* L.) and related species (Konarev, 1995). Polymorphic TSI was found (using gelatin replicas method) to inhibit extracellular proteinase of pathogenic for sunflower fungi *Sclerotinia sclerotiorum* (Konarev et al., 1999b) and probably belong to potato inhibitor I family (Konarev et al., 2000, 2002a). The trypsin inhibitor was difficult to analyze because it could be only detected by the gelatin replica method but was not visible on SDS-PAGE until it was linked to PAG by glutaraldehyde. The inhibitor was purified by affinity chromatography on trypsin-Sepharose followed by reverse phase HPLC. SDS-PAGE and mass-spectrometry showed that it was extremely low-molecular weight peptide (1, 4 kDa) (Konarev et al., 1998, 1999a, 2000) whose amino acid sequence could not be determined for some time. It turned to be a cyclic peptide, with no free N-terminus (Luckett et al., 1999). The analysis revealed that this inhibitor called SFTI-1 corresponds by sequence to a loop (containing a reactive centre) of well known and widely distributed Bowman-Birk inhibitors which in sunflower exists independently of the rest part of the inhibitor molecule being in the cyclic form (Luckett et al., 1999) that was later confirmed by other researchers (Korsinczky et al., 2001 & 2004; Mylne et al., 2011). This similarity can also be result of convergence. SFTI-1 was found only in *Helianthus* and related *Tithonia* species and not in any studied asterids including Compositae (Konarev et al., 2000, 2002a, 2004). SFTI-1 is considered as the most potent naturally occurring plant Bowman-Birk inhibitor known so far (Korsinczky et al., 2004). More than one hundred publications on SFTI-1 have appeared since 1999. It was revealed that the inhibitor was extremely robust, resistant to proteolysis (no N- or C-ends) which, coupled to its high potency of inhibition for such proteinases as matrilipase, thrombin, kallikrein 4 etc. made it an excellent candidate for use as a template for further development of drugs for therapy against cancer, thromboembolism and other proteinase-related human pathologies (Li, et al., 2007; Luckett et al., 1999; Swedberg et al., 2009; Białas & Kafarski, 2009) or agents for plant protection against pests and pathogens.

3.2.4 Glutenin-specific proteinase of sunn pest *Eurygaster integriceps* Put. responsible for wheat gluten degradation

Sunn pest *E. integriceps* and related wheat bugs cause huge losses in grain quality in Russia, South and South-East Europe, Middle East and Central Asia. The main damaging agent is

the proteinase of the salivary glands which is injected into maturing wheat grains for extraintestinal digestion. The traces of enzyme remaining in the grains after the bug's attack show activity in dough mixing before baking even several years later. The proteinase degrades the wheat gluten proteins, leading to a loss of gluten and dough viscoelasticity and poor processing properties. The sunn pest proteinase was studied during the last decades in many laboratories but attempts to purify it failed probably due to narrow substrate specificity, low sensitivity to known proteinase inhibitors (which prevented the use of affinity chromatography), instability in purified form and lack of suitable methods for its detection at purification. We have developed method applicable for detection of sunn pest proteinase in protein extracts both from salivary glands and damaged seeds based on using of IEF in combination with thin layer of insoluble in acetic acid glutenin (protein determining quality of gluten and substrate for bug's proteinase) attached to supporting plastic film. In order to make it possible to use affinity chromatography on immobilized potato chymotrypsin inhibitor I at its low affinity to the enzyme, conditions of fractionation were modified so that proteinase left linked to inhibitor until elution. It was found that enzyme binds to the inhibitor at high pH (about 10) that made it possible washing column after loading just by solvent used for sample. The common washing by buffers with lower pH was omitted and proteinase began to elute after replacement of solvent by water. The elution continued after changing water to 0.01M HCl. Both fractions gave in SDS-PAGE the same almost pure proteinase band with traces of low-molecular proteins which can be easily removed by gel-filtration. The absence of defined elution peak can be explained by low affinity of interaction. IEF followed by detection using glutenin film and SDS-sedimentation methods confirmed proteinase nature of isolated fraction. The addition to all solutions of 0.01% non-ionic detergent Triton X-100 improved stability of proteinase after purification. This 28 kDa protein was partially sequenced by mass spectrometry and Edman degradation which showed homology to serine proteases from various insects. Three full length clones were obtained from cDNA isolated from sunn pest salivary glands using degenerate PCR based on the sequences obtained. The cleavage site of the protease was determined using recombinant and synthetic peptides and shown to be between the consensus hexapeptide and nonapeptide repeat motifs present in the high molecular weight subunits of wheat glutenin. Homology models were generated for one of the proteinase isoforms identified in this study. The novel specificity of this protease and data obtained may find various applications in both fundamental and applied studies, e.g. in design of effective inhibitors for improving wheat resistance to pests and limiting proteinase activity in food technologies. Besides, the proteinase cleaves one of epitopes of glutenin defined by Wal et al (1999) as minimal epitope for the HLA-DQ8 form of celiac disease, so probably it can be used for gluten proteins modification to decrease their toxicity and autoimmune activity for gluten-sensitive people (Konarev et al., 2011).

4. Conclusion

The proposed set of universal and sensitive methods for detection among plant proteins of insect, mammalian, fungal, bacterial and plant α -amylase and proteinase inhibitors and also these hydrolases themselves significantly simplify the analysis of inhibitor polymorphism and the search for novel forms of enzymes and their inhibitors. The detection methods enhance the capacity of affinity chromatography, mass-spectrometry and other techniques of protein fractionation and characterisation. They may be used in a wide variety of fields to answer

many questions from plant systematics to the identification of proteins with potential uses in plant resistance as well as medicine. The further improvement in resolution, sensitivity and specificity of these techniques will help increase the efficiency of mentioned research.

5. Acknowledgment

These above described studies were carried out according to the Program of Fundamental Investigations on Plant Protection of the Russian Academy of Agricultural Sciences and were supported by the following grants from: The Russian Foundation for Basic Research (93-04-21290-a); Japan International Research Center for Agricultural Sciences (1997-1998); The Royal Society Joint Project "Characterization of Sunflower Seed Proteins" (683072.P810); an "Ex-Agreement Visit" Grant from The Royal Society of London (2002); a Fellowship Grants from Rothamsted International (2005) and the BBSRC Underwood fund (2006). Rothamsted Research receives grant-aided support from the Biotechnology and Biological Sciences Research Council of the United Kingdom. Authors are very grateful to Prof. Peter Shewry for continuing support and encouragement over the years of these projects. The authors would also like to thank all their colleagues who participated in the research.

6. References

- Białas, A. & Kafarski, P. (2009). Proteases as anti-cancer targets - molecular and biological basis for development of inhibitor-like drugs against cancer. *Anticancer Agents Med. Chem.*, Vol.9, No.7, pp. 728-762, ISSN 1871-5206
- Buonocore, V.; Petrucci, T. & Silano V. (1977). Wheat protein inhibitors of α -amylase. *Phytochemistry*, Vol.16, No.7, pp. 811-820, ISSN 0031-9422
- Buonocore, V.; Poerio, E.; Gramenzi, F. & Silano, V. (1975). Affinity column purification of amylases on protein inhibitors from wheat kernel. *Journal of Chromatography*, Vol.114, No1., pp.109-114, ISSN 0021-9673
- Buonocore, V.; Poerio, E.; Gramenzi, F. & Silano, V. (1975). Affinity column purification of amylases on protein inhibitors from wheat kernel. *Journal of Chromatography*, Vol.114, No1. pp. 109-114, ISSN 0021-9673
- Burger, W. C. & Schroeder, R. L. (1976). A sensitive method for detecting endopeptidases in electrofocused thin-layer gels. *Analytical Biochemistry*, Vol.71, No.2, pp. 384-392, ISSN 0003-2697
- Chagot, B.; Pimentel, C.; Dai, L.; Pil, J.; Tytgat, J.; Nakajima, T.; Corzo, G.; Darbon, H. & Ferrat, G. (2005) An unusual fold for potassium channel blockers: NMR structure of three toxins from the scorpion *Opisthacanthus madagascariensis*. *Biochem J.* Vol. 388, No.1, pp. 263-71, ISSN 0264-6021
- Connors, R.; Konarev, Al.V.; Forsyth, J.; Lovegrove, A.; Marsh, J.; Joseph-Horne, T.; Shewry, P. & Brady, R. L. (2007). An unusual helix-turn-helix protease inhibitory motif in a novel trypsin inhibitor from seeds of veronica (*Veronica hederifolia* L.). *The Journal of Biological Chemistry*, Vol.282, No.38, pp. 27760-27768, ISSN 0021-9258
- Dunaevsky, Y. A.; Elpidina, E.; Vinokurov, K.; Belozersky, M. (2005). Protease inhibitors in improvement of plant resistance to pathogens and insects. *Molecular Biology*, Vol.39, No.4, pp. 608-613, ISSN 0026-8933

- Fontanini, D.; Capocchi, A.; Saviozzi, F. & Galleschi, L. (2007). Simplified electrophoretic assay for human salivary alpha-amylase inhibitor detection in cereal seed flours. *J Agric Food Chem.*, Vol.55, No.11, pp. 4334-4339, ISSN 0021-8561
- Franco, O.L.; Rigden, D.J.; Melo, F.R. and Grossi-De-Sá, M.F. (2002). Plant alpha-amylase inhibitors and their interaction with insect alpha-amylases. *Eur. J. Biochem.*, (January), Vol.269, No.2, pp. 397-412, ISSN 0014-2956
- Gatehouse, J.A. (2011). Prospects for using proteinase inhibitors to protect transgenic plants against attack by herbivorous insects. *Curr. Protein. Pept. Sci.*, (Aug 1), Vol.12, No.5, pp. 409-16, ISSN 1389-2037
- Giri, A.P. & Kachole, M. S. (1996). Detection of electrophoretically separated amylase inhibitors in starch-polyacrylamide gels. *Journal of Chromatography*, Vol.752, No.1-2, pp. 261-264, ISSN 0021-9673
- Habib, H. & Fazili, K. M. (2007). Plant protease inhibitors; a defence strategy in plants. *Biotechnology and Molecular Biology Review*, Vol.2, No.3, (August 2007), pp. 68-85, ISSN 1538-2273
- Harsulkar, A.M.; Giri, A.P.; Gupta, V.S.; Sainani, M.N.; Deshpande, V.V.; Patankar, A.G. & Ranjekar, P.K. (1998). Characterization of *Helicoverpa armigera* gut proteinases and their interaction with proteinase inhibitors using gel X-ray film contact print technique. *Electrophoresis*, Vol.19, No.8-9, (Jun), pp. 1397-402, ISSN 1522-2683.
- Hejgaard, J. (1981). Isoelectric focusing of subtilisin inhibitors. Detection and partial characterisation of cereal inhibitors of chymotrypsin and microbial proteases. *Analytical Biochemistry*, Vol.116, No.2. P.444-449, ISSN 0003-2697
- Konarev, Al.V. & Fomicheva, Yu.V. (1991a). Cross analysis of the interaction of alpha-amylase and proteinase components of insects with protein inhibitors from wheat endosperm. *Biokhimiya (Biochemistry, Moscow)*. Vol.56. No.4, pp. 628-638, ISSN 0006-2979
- Konarev, Al.V. & Fomicheva, Yu.V. (1991b). Hydrolase inhibitors and coevolution of cereals and harmful insects. *Proceedings of IX All-Union Conference on Plant immunity to diseases and pests*, pp. 270-271, Minsk, USSR (In Russian)
- Konarev, Al.V. & Fasulati, S.R. (1996). Proteinases of colorado potato beetle and their plant proteinaceous inhibitors. *Proceedings of XX Int. Congr.of Entomology*, Firenze, Italy, August 25-31, 1996
- Konarev, Al.V. & Zoteeva, N.M. (2006). Induced by phytophthora infection accumulation of proteinase inhibitors in potato varieties. *Proceedings of All-Russia scientific-practical conference " Induced immunity of agricultural crops as important direction in plant protection"*, p.45-47, Bolshie Vyazemy, Moskovskaya oblast., 15-16 Nov, 2006. (in Russian)
- Konarev, Al.V. (1981). Specificity of wheat grain albumins (alpha-amylase inhibitors) towards enzymes of sunn pest (*Eurygaster integriceps* Put.). *Bulletin VIR*, Vol. 114., pp.30-32, ISSN 0202-5361 (In Russian)
- Konarev, Al.V. (1982a). Component composition and genetic control of insect alpha-amylase inhibitors from wheat and aegilops grain. *Doklady VASKhNIL*, No.6. P. 42-44, ISSN 0042-9244 (In Russian)
- Konarev, Al.V. (1982b). Identification of inhibitors of endogenous and exogenous alpha-amylases among wheat proteins. *Bulletin VIR*. V. 118.P. 11-12, ISSN 0202-5361 (In Russian)

- Konarev, A.I.V. (1984). Identification of inhibitors of thiol proteinases of insects and grain in wheat and other cereals. *Doklady VASKhNIL*, No.10, pp. 13-15, ISSN 0042-9244 (In Russian)
- Konarev, A.I.V. (1985) Methods for analyzing the component composition of cereal alpha-amylase and proteinase inhibitors. *Prikladnaya Biokhimiya i Microbiologia (Applied Biochemistry and Microbiology, Moscow)*, Vol.21, No.1, pp. 92-100, ISSN, 0555-1099
- Konarev, A.I.V. (1986a). Analysis of proteinase inhibitors from wheat grain by gelatin replicas method. *Biokhimiya (Biochemistry, Moscow)*, Vol.51, No. 2, pp. 195-201, ISSN 0006-2979
- Konarev, A.I.V. (1986b). Relationship and variability of proteinase and amylase inhibitors in wheat and related cereals. *Agricultural Biology*, No.3., pp. 46-52, ISSN 0131-6397 (In Russian)
- Konarev, A.I.V. (1987a). Component composition of trypsin inhibitors from grain and leaves of wheat. *Doklady VASKhNIL*, No.12, pp. 6-9, ISSN 0042-9244 (In Russian)
- Konarev, A.I.V. (1987b). Variability of trypsin-like proteinase inhibitors in wheat and related cereals. *Agricultural Biology*, No.5, pp. 17-24, ISSN 0131-6397 (In Russian)
- Konarev, A.I.V. (1988). Polymorphism of chymotrypsin-subtilisin inhibitors and possibilities of its use in wheat variety identification. In: *Biochemical identification of varieties. Materials of III International symposium ISTA*, V. Konarev & I. Gavriljuk (Ed.), pp. 176-181, VIR, Leningrad, USSR
- Konarev, A.I.V. (1989). The evolutionary variability and genetic control of chymotrypsin-subtilisin inhibitors in wheat. *Doklady VASKhNIL*, No.5, pp. 8-10, ISSN 0042-9244 (In Russian)
- Konarev, A.I.V. (1990a). Cross analysis of interaction of endogenous alpha-amylase and proteinase components with protein inhibitors in wheat. *Doklady VASKhNIL*. No.1, pp. 10-13, ISSN 0042-9244 (In Russian)
- Konarev, A.I.V. (1990b). Detection of lectins in analytical isoelectrofocusing of wheat proteins. *Biokhimiya (Biochemistry, Moscow)*, Vol. 55, No.11, pp. 1975-1983, ISSN 0006-2979
- Konarev, A.I.V. (1992a). Analysis of native trypsin inhibitors from endosperm of wheats with various genome composition. *Agricultural Biology*, No.5, pp. 10-16, ISSN 0131-6397 (In Russian)
- Konarev, A.I.V. (1992b). Systems of hydrolase inhibitors in cereals: organisation, functions and evolutionary variability. *Doctor of Biological Sciences Dissertation*, Scientific Council in the Russian Academy of Sciences A.N.Bach Institute of Biochemistry, 356 p., Moscow, Russia (In Russian)
- Konarev, A.I.V. (1993). Component composition, genome and chromosome control of trypsin inhibitors from bread wheat leaves, roots and germs. *Agricultural Biology*, No.1., pp. 43-52, ISSN 0131-6397 (In Russian)
- Konarev, A.I.V. (1995). Proteinaceous hydrolase inhibitors in mechanisms of plant defense. *Proceedings of 1st All-Russia Plant Protection Congress*, pp. 205-206, VIZR, St. Petersburg, Russia (December 4-9, 1995) (In Russian)
- Konarev, A.I.V. (1996). Interaction of insect digestive enzymes with protein inhibitors from plants and host-parasite coevolution. *Euphytica*, Vol.92, No.1-2, pp. 89-94, ISSN 0014-2336

- Konarev, Al.V. (2000a). Insect and fungal enzyme inhibitors in study of variability, evolution and resistance of wheat and other Triticeae Dum. cereals. In: *Wheat Gluten*, P.R. Shewry & A.Tatham, (Ed.), 526-530, ISBN 0-85404-865-0, Royal Society of Chemistry, Cambridge, UK
- Konarev, Al.V. (2000b). Proteinase inhibitors and resistance of potato to Colorado potato beetle. In "*Modern systems of plant protection and novel approaches to improving of potato resistance to Colorado potato beetle*", K.G. Skryabin & K.V.Novozhilov (Ed.), pp.35-40, Nauka, Moscow, Russia, ISBN 5-02-005214-0 (In Russian)
- Konarev, Al.V.; Anisimova, I.N.; Gavrilova, V.A. & Shewry, P.R. (1999a). Polymorphism of inhibitors of hydrolytic enzymes present in cereal and sunflower seeds. In: *Genetics and breeding for crop quality and resistance*, G.T.S. Mugnozza, E.Porceddu & M.A.Pagnotta, (Ed.), 135-144, ISBN 0792358449, Kluwer Academic Publishers, Dordrecht, NL
- Konarev, Al.V.; Kochetkov, V.V.; Bailey, J.A. & Shewry, P.R. (1999b). The detection of inhibitors of the *Sclerotinia sclerotiorum* (Lib) de Bary extracellular proteinases in sunflower, *J. Phytopathology*, Vol.147, No.2, pp. 105-108, ISSN 0931-1785
- Konarev, Al.V.; Tomooka, N.; Ishimoto M. & Vaughan, D.A. (1999c). Variability of the inhibitors of serine, cysteine proteinases and insect alpha-amylases in *Vigna* and *Phaseolus*. In: *Genetics and breeding for crop quality and resistance*, G.T.S.Mugnozza, E.Porceddu & M.A.Pagnotta (Ed.), 173-181, ISBN 0792358449, Kluwer Academic Publishers, Dordrecht, NL
- Konarev, Al.V.; Anisimova, I.N.; Gavrilova, V.A.; Rozhkova, V.T.; Fido, R.; Tatham, A.S. & Shewry, P.R. (2000). Novel proteinase inhibitors in seeds of sunflower (*Helianthus annuus* L.): polymorphism, inheritance and properties. *Theoretical and Applied Genetics*, Vol.100, No.1, pp. 82-88, ISSN 0040-5752
- Konarev, Al.V.; Anisimova, I.N.; Gavrilova, V.A.; Vachrusheva, T.E.; Konechnaya, G.Yu.; Lewis, M. & Shewry, P.R. (2002a). Serine proteinase inhibitors in Compositae: distribution, polymorphism and properties. *Phytochemistry*, Vol.59, No. 3, pp. 279-291, ISSN 0031-9422
- Konarev, Al.V.; Tomooka, N. & Vaughan, D.A. (2002b). Proteinase inhibitor polymorphism in the genus *Vigna* Savi subgenus *Ceratotropis* and its biosystematic implications. *Euphytica*, Vol., 123, No.2, pp. 165-177, ISSN 0014-2336
- Konarev, Al.V.; J.Griffin; Konechnaya, G.Yu.& Shewry, P.R. (2004). The distribution of serine proteinase inhibitors in seeds of the Asteridae. *Phytochemistry*, Vol.65, No.2, pp. 3003-3020, ISSN 0031-9422
- Konarev, Al.V.; Lovegrove, A. & P.R.Shewry. (2008). Serine proteinase inhibitors in seeds of *Cycas siamensis* and other gymnosperms. *Phytochemistry*, Vol.69, No. 13, pp.2482-2489, ISSN 0031-9422
- Konarev, Al.V.; Lovegrove, A. & Shewry P. R. (2009). Inhibitors of microbial serine proteinases in cycads and other gymnosperms. *Proceedings of XIV International Congress on Molecular Plant-Microbe Interactions (IS-MPMI)*, p.20, Toronto, Canada, July 19-23
- Konarev, Al.V.; Beaudoin F.; Marsh, J.; Vilkova, N.A.; Nefedova, L.I.; Sivri, D.; Koxsel, H.; Shewry, P.R. & Lovegrove, A. (2011). Characterization of a glutenin-specific serine proteinase of sunn bug *Eurygaster integriceps* Put. *Journal of Agricultural and Food Chemistry*. Vol.59, No.6, pp. 2462-2470, ISSN 0021-8561

- Konarev, V.G. (1996). Problems of species and genome in plant breeding. In: Molecular biological aspects of applied botany, genetics and plant breeding. Theoretical bases of plant breeding, A.V.Konarev (Ed.), pp. 14-24, VIR, St. Petersburg, Russia (In English)
- Korsinczky, M.L.; J.; Schirra, H.J.; Rosengren, K.J.; West, J.; Condie, B.A.; Otvos, L.; Anderson, M.A. & Craik, D.J. (2001). Solution structures by ¹H NMR of the novel cyclic trypsin inhibitor SFTI-1 from sunflower seeds and an acyclic permutant. *Journal of Molecular Biology*, Vol.311, No. 3, pp. 579-591, ISSN 0022-2836
- Korsinczky, M.L.; Schirra, H.J, & Craik, D.J. (2004). Sunflower trypsin inhibitor-1. *Curr Protein Pept Sci*. Vol.5, No.5, pp. 351-364, ISSN 1389-2037
- Li, P.; Jiang, S.; Lee, S.-L.; Lin, C.Y.; Johnson, M.D.; Dickson, R.B.; Michejda, C.J. & Roller, P.P. (2007). Design and synthesis of novel and potent inhibitors of the type II transmembrane serine protease, matriptase, based upon the sunflower trypsin inhibitor-1. *J. Med. Chem.*, Vol.50, No.24, pp. 5976-5983, ISSN 0022-2623
- Luckett, S.; Garcia, R.S.; Barker, J.J.; Konarev, A.I.V.; Shewry, P.R.; Clarke, A.R. & Brady R.L. (1999). High-resolution structure of a potent, cyclic proteinase inhibitor from sunflower seeds. *Journal of Molecular Biology*, Vol.290, No.2., (July), pp. 525-533, ISSN 0022-2836
- Möller, C.; Rahmankhah, S.; Lauer-Fields, J.; Bubis, J.; Fields, G.B. & Mari, F. (2005). A novel conotoxin framework with a helix-loop-helix (Cs α/α) fold. *Biochemistry*, Vol.44, No.49, (November 16), pp. 15986-15996, ISSN 0006-2960
- Mosolov, V.V. & Valueva, T.A. (2005) Proteinase inhibitors and their function in plants: A review. *Applied Biochemistry and Microbiology*, Vol.41, No.3, pp. 227-246, ISSN 0003-6838
- Mulimani, V.H.; Sudheendra, K. & Giri, A.P. (2002). Detection of legume protease inhibitors by the Gel-X-ray film contact print technique. *Biochemistry and Molecular Biology Education*, Vol.30, No.1, pp. 40-44, ISSN 1539-3429
- Mylne, J.S.; Colgrave, M.L.; Daly, N.L.; Chanson, A.H.; Elliott, A.G.; McCallum, E.J.; Jones A. & Craik D.J. (2011). Albumins and their processing machinery are hijacked for cyclic peptides in sunflower. *Nat Chem Biol.*, Vol.7, No.5, (May), pp.257-259, ISSN 1552-4450
- Nagaraj, R.H. & Pattabiraman, T.M. (1985). Isolation of an amylase inhibitor from *Setaria italica* grains by affinity chromatography on blue-sepharose and its characterization. *J. Agric. Food Chem.*, Vol.33, No.4, pp. 646-650, ISSN 0021-8561
- Nolde, S..B.; Vassilevski, A.A.; Rogozhin, E.A.; Barinov, N.A.; Balashova, T.A.; Samsonova, O.V.; Baranov, Y.V.; Feofanov, A.V.; Egorov, T.A.; Arseniev, A.S. & Grishin, E.V. (2011). Disulfide-stabilized helical hairpin structure and activity of a novel antifungal peptide EcAMP1 from seeds of barnyard grass (*Echinochloa crus-galli*). *J Biol Chem*; Vol.286, No.28, (July 15), pp. 25145-25153, ISSN 0021-9258
- Park, S.S.; Abe, K.; Kimura, M.; Urisu, A. & Yamasaki, N. (1997). Primary structure and allergenic activity of trypsin inhibitors from the seeds of buckwheat (*Fagopyrum esculentum* Moench). *FEBS Letters*, Vol.400, No.1, pp. 103-107, ISSN 0014-5793
- Pichare, M.M. & Kachole M.S. (1994). Detection of electrophoretically separated protease inhibitors using X-ray film. *Journal of Biochemical and Biophysical Methods*, Vol.28, No.3, (April 1994), pp. 215-224, ISSN 0165-022X

- Saxena, L.; Iyer B.K. & Ananthanarayan, L. (2010). Purification of a bifunctional amylase/protease inhibitor from ragi (*Eleusine coracana*) by chromatography and its use as an affinity ligand. *J. Chromatogr. B Analyt. Technol. Biomed. Life. Sci.*, Vol.878, No.19. pp. 1549-1554, ISSN 1570-0232
- Shewry, P. R. & Lucas, J.A. (1997) Plant proteins that confer resistance to pests and pathogens. *Advances in Botanical Research*, Vol.26, pp. 135-192, ISSN 0065-2296
- Shewry, P.R.; Halford, N.G. & Lafiandra, D.(2003). Genetics of wheat gluten proteins. *Advances in Genetics*, Vol.49, pp. 111-184, ISSN 0065-2660
- Swedberg, J.E.; Nigon, L.V.; Reid, J.C.; de Veer, S.J.; Walpole, C.M.; Stephens, C.R.; Walsh, T.P.; Takayama, T.K.; Hooper, J.D.; Clements, J.A.; Buckle, A.M. & Harris, J.M. (2009). Substrate-guided design of a potent and selective kallikrein-related peptidase inhibitor for kallikrein 4. *Chemistry & Biology*, Vol.16, No.6, pp. 633-643, ISSN 1074-5521
- Van de Wal, Y.; Kooy, Y. M. C.; van Veelen, P.; Vader, W.; August, S. A.; Drijfhout, J. W.; Pena, S. A. & Koning, F. (1996). Glutenin is involved in the gluten-driven mucosal T cell response. *Eur. J. Immunol.*, Vol. 29, No.10, pp. 3133-3139, ISSN 0014-2980