# STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF THE SECOND KUNITZ-TYPE PEPTIDASE INHIBITOR DOMAIN OF THE HUMAN WFIKKN1 PROTEIN

Ph.D. Thesis

**Alinda Nagy**

Faculty of Science of Eötvös Loránd University,
Doctorate School in Biology, Classical and Molecular Genetics Ph.D. Program

Supervisor: Prof. László Patthy

Institute of Enzymology, Biological Research Center, Hungarian Academy of Sciences

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# INTRODUCTION

Following the completion of the Human Genome Project a fundamental aim of biological research is the identification of all of the human genes, *via* analyses of the sequence of the genome, and the subsequent characterization of the structure, molecular function and biological role of the encoded proteins. Inhibitors controlling the activity of peptidases are a group of great relevance to biology, medicine and drug development, since they play a role in the onset of a number of diseases.

In 2001, using *in silico* methods the Functional Genomics Group of the Institute of Enzymology, BRC, HAS, identified the *WFIKKN1* gene on human chromosome 16 that encodes a secreted multidomain protein. The WFIKKN1 protein contains a WAP-domain, a Follistatin-domain, an Immunoglobulin-domain, two Kunitz-domains and an NTR-domain. The function of the newly identified protein is unknown, but it consists of domains which frequently participate in the inhibition of various serine- and metallo-peptidases. On the basis of homology it was assumed that the WFIKKN1 protein is a multivalent peptidase inhibitor.

In 2002, the Functional Genomics Group identified on human chromosome 17 the gene encoding the WFIKKN2 protein that has the same domain organisation as the WFIKKN1 protein. In view of the equivalent domain structure it was hypothesized that the WFIKKN2 protein is also a multivalent peptidase inhibitor. The tissue expression pattern of the two proteins, however, is markedly different suggesting that they have distinct biological roles. Whereas the *WFIKKN1* gene is expressed primarly in adult pancreas, liver and thymus, and not in brain or ovary, the most significant expression of the *WFIKKN2* gene is observed in ovary, testis and brain, with no expression in liver.

# OBJECTIVES

Although the biological role of the WFIKKN1 protein is unknown, based on its domain structure we presumed that it is an inhibitor able to control the activity of different peptidases. The aim of our research is the structural and functional characterization of the human WFIKKN1 protein and the identification of its target peptidases using the recombinant peptidase inhibitor modules of the protein.

Within this research the aim of my work was to carry out the structural and functional characterization of its second Kunitz-type peptidase inhibitor domain, in order to better understand the biological role and medical relevance of the WFIKKN1 protein.

Major goals of the work:

1. Expresssion of the recombinant WFIKKN1-KU2 domain.
2. Study of the inhibitor activity of the recombinant WFIKKN1-KU2 domain and the identification of its target peptidases.
3. Determination of the 3D structure at the atomic level of the recombinant WFIKKN1-KU2 domain using NMR spectroscopy.
4. Analyses of the structural and functional properties of the recombinant WFIKKN1-KU2 domain to obtain information on the biological role of the WFIKKN1 protein.

# METHODS

**Cloning**: The pMed23 and pPICZαA expression vector contructions containing the DNA segment coding for the WFIKKN1-KU2 domain were created with standard methods of recombinant DNA technology.

**Expression and purification of the proteins:** The recombinant protein was expressed in *Escherichia coli* JM109 and *Pichia pastoris* GS115 cells. The proteins were purified by gel filtration, and nickel and trypsin-Sepharose affinity chromatography.

**Protein sequencing (**Dr. András Patthy, Gödöllő): The N-terminal sequencing of the purified proteins was performed with a PE-Applied Biosystems Ltd Procise protein sequencing system.

**Tricine/SDS polyacrilamid gel electrophoresis:** The composition of protein samples was analysed using 16%-os slab gels under both reducing and nonreducing conditions.

**Spectrophotometry:** The concentration of the recombinant WFIKKN1-KU2 domain was determined spectrophotometrically using the extinction coefficient 14000 $M^{-1} \cdot cm^{-1}$.

**Circular Dichroism (CD) spectroscopy:** The CD spectra of the recombinant protein were measured over the range of 190-250 nm using a JASCO J-720 spectropolarimeter. The measurements were carried out in protein solutions of 0.1 mg/ml in 10 mM Tris/HCl pH 8.0 buffer, at 25 $^{o}$C. Secondary structure of the protein was estimated from the CD spectra with the CDPro software. Thermal unfolding of the protein was monitored at 203 nm, in the range of 40-90 $^{o}$C. Melting temperature was determined by derivative processing of changes in CD using the spectrum analysis program of the spectropolarimeter.

**Enzyme kinetics studies:** The peptidase inhibitory effect of the recombinant WFIKKN1-KU2 was measured against trypsin, elastase, chymotrypsin, plasmin, thrombin, tissue plaminogen activator, plasma kallikrein, pancreatic kallikrein, lung tryptase and urokinase. The activity of the proteases on synthetic peptide-pNA substrates was monitored spectrophotometrically using a Cary 300 Scan spectrophotometer at 410 nm, 37 $^{o}$C ($\Delta\varepsilon$=8800 $M^{-1}cm^{-1}$), and the initial rates of the reaction were determined. The dissociation

constant of the trypsin-inhibitor complex, $K_i$ was determined from plotting of the apparent $K_m$ values against the inhibitor concentration at which they were obtained.

**Sequence analyses:** Multiple alignments of the amino acid sequences of Kunitz-domains were constructed using CLUSTAL W.

**Nuclear Magnetic Resonance (NMR) studies** (Gottfried Ötting, Canberra)**:** NMR spectra of the recombinant WFIKKN1-KU2 protein were recorded using 1.1 mM solutions in 90% $H_2O$/10% $D_2O$ on a Varian Inova 800 MHz NMR spectrometer, at pH 4.5 and 25 $^oC$. The NMR structure of the domain was calculated using the program DYANA; the conformers were energy minimised in water using the program OPAL; the Ramachandran plot was analysed using PROCHECK-NMR; RMSD (Root Mean Square Deviation) values were calculated using the program MOLMOL.

## RESULTS AND DISCUSSION

### 1. Structural characterization of the recombinant WFIKKN1-KU2 domain

1.1. Based on the CD spectra of the WFIKKN1-KU2 domain we have established that the domain has secondary structure elements similar to those of other members of the Kunitz-domain family. The thermal stability of the recombinant domain is lower than that of other Kunitz-type domains: it has a $T_m$ value of 61 $^oC$.

1.2. The results of the NMR studies have shown that the structure of the WFIKKN1-KU2 domain is very similar to that of BPTI, with a backbone RMSD of 0.9 Å between the structures of the two Kunitz-domains. In particular, the peptidase-binding loops of the two proteins are very similar. Thus the NMR studies have shown that the structure of the KU2 domain is closely related to those of the Kunitz-type peptidase inhibitors.

## 2. Functional characterization of the recombinant WFIKKN1-KU2 domain

2.1. The enzyme kinetics studies have shown that the WFIKKN1-KU2 domain is inhibitor of trypsin. The dissociation contant for its complex with trypsin ($K_i$) is 9.6 x $10^{-9}$ M which is about five magnitudes lower than that of BPTI ($K_i$ = 1.6 x $10^{-13}$ M). The affinity of WFIKKN1-KU2 toward trypsin is weaker than that of other Kunitz-type inhibitors for their target peptidases, as they usually form very tight complexes with binding constants in the picomolar range with the inhibited peptidases.

2.2. The WFIKKN1-KU2 domain was found to show remarkable specificity for trypsin. When the inhibitor was employed at 1 μM final concentration, complete inhibition of trypsin (30 nM initial concentration) was achieved, but no detectable inhibition was observed in the case of plasmin, lung tryptase, plasma kallikrein, thrombin, urokinase, tissue plasminogen activator, pancreatic kallikrein, chymotrypsin or elastase serine peptidases with initial enzyme concentration similar to that of trypsin. Such a marked trypsin specificity is unusual among Kunitz-type domains. Typically they display much broader specificity and inhibit various peptidases including trypsin.

## 3. Analyses of the structure-function relationships of the recombinant WFIKKN1-KU2 domain

3.1. Comparison of the 3D structure of the WFIKKN1-KU2 domain with those of other Kunitz-type inhibitor-trypsin complexes has revealed that the side-chain of the bulky Trp22 at the P2' site of the WFIKKN1-KU2 interferes with the formation of the strong interactions typically found between peptidases and their inhibitors. It is likely that the unfavourable side-chain conformations of Trp22 are responsible for the lower trypsin affinity of the WFIKKN1-KU2 domain.

3.2. In the KU2 domain of the macaque, mouse, rat, bovine, dog and chicken WFIKKN1 protein the P1 residue is a Gln. Gln instead of Lys at the P1 site in BPTI reduces the association with trypsin by almost five orders of magnitude, indicating that the Gln residue in the WFIKKN1-KU2 domain would largely destroy trypsin inhibition. Among all Kunitz-type inhibitors for which 3D structures have been determined, only the Kunitz-type domain of β2 bungarotoxin has a Gln at the P1 site. The β bungarotoxin is a heterodimer neurotoxin, the II chain of which is not a peptidase inhibitor but a K+-channel binding unit.

## CONCLUSIONS

In the course of the functional studies, the human WFIKKN1-KU2 domain was found to show remarkable specificity for trypsin but our results (2.1 and 3.2) suggest that inhibition of trypsin or other trypsin-like serine peptidases may not be the most relevant aspect of the biological role of the domain. In 2003 J. Hill *et al.* demonstrated that the mouse orthologue of the human WFIKKN2 protein inhibits the activity of the mature myostatin. Myostatin is a muscle-specific member of the TGFß superfamily that plays an important role in the negative regulation of muscle development.

In view of the results of the structural and functional studies of the human WFIKKN1-KU2 domain and the observed myostatin-binding function of the WFIKKN2 protein we reject the hypothesis (based on the domain composition of the protein), that the main function of the WFIKKN1 protein is the inhibition of various peptidases. It seems more likely that, like the homologue WFIKKN2 protein, it plays a role controlling the activity of a protein belonging to the TGFβ growth factor family.

# PUBLICATIONS

## Publications underlying the dissertation

**Nagy, A.,** Trexler, M. and Patthy, L. (2003) Expression, purification and characterization of the second Kunitz-type protease inhibitor domain of the human WFIKKN protein. *Eur. J. Biochem.* **270**, 2101-7.

Liepinsh, E., **Nagy, A**., Trexler, M., Patthy, L. and Otting, G. (2006) Second Kunitz-type protease inhibitor domain of the human WFIKKN1 protein. *J. Biomol. NMR* **35**, 73-8.

## Publications not tightly connected with the topic of the dissertation

Tordai, H., **Nagy, A**., Farkas, K., Banyai, L. and Patthy, L. (2005) Modules, multidomain proteins and organismic complexity. *FEBS J.* **272**, 5064-78.

Tress, M.L., Martelli, P.L., Frankish, A., Reeves, G., Wesselink, J.J., Yeats, C., Olason, P.I., Albrecht, M., Hegyi, H., Giorgetti, A., Raimondo, D., Lagarde, J., Laskowski, R., Lopez, G., Sadowski, M.I., Watson, J., Fariselli, P., Rossi, I., **Nagy, A**., Kai, W., Størling, Z., Orsini, M., Assenov, Y., Blankenburg, H., Huthmacher, C., Ramirez, F., Schlicker, A., Denoued, F., Jones, P., Kerrien, S., Orchard, S., Birney, E., Brunak, S., Casadio, R., Guigo, R., Harrow, J., Hermjakob, H., Jones, D.T., Lengauer, T., Orengo, C., Patthy, L., Thornton, J., Tramontano, A. and Valencia, A. (2007) The implications of alternative splicing in the ENCODE protein complement. *Proc. Natl. Acad. Sci. USA* (in press)