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Expression, purification and characterization of the second Kunitz-type protease inhibitor domain of the human WFIKKN protein

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Recently we have described a novel secreted protein (the WFIKKN protein) that consists of multiple types of protease inhibitory modules, including two tandem Kunitz-type protease inhibitor-domains. On the basis of its homologies we have suggested that the WFIKKN protein is a multivalent protease inhibitor that may control the action of different proteases. In the present work we have expressed the second Kunitz-type protease inhibitor domain of the human protein WFIKKN in *Escherichia coli*, purified it by affinity chromatography on trypsin-Sepharose and its structure was characterized by CD spectroscopy. The recombinant protein was found to inhibit trypsin ($K_i = 9.6$ nM), but chymotrypsin, elastase, plasmin, pancreatic kallikrein, lung tryptase, plasma kallikrein, thrombin, urokinase or tissue plasminogen activator were not inhibited by the recombinant protein even at 1 μM concentration. In view of the marked trypsin-specificity of the inhibitor it is suggested that its physiological target may be trypsin.

Keywords: Kunitz-domain; multidomain protease inhibitor; serine proteinases; trypsin.

Recently we have identified two closely related human proteins (WFIKKN and WFIKKNRP) each of which contain a WAP-domain, a Follistatin/Kazal domain, an Immunoglobulin-domain, two Kunitz-domains and an NTR-domain [2,3]. The tissue expression pattern of the two proteins, however, is markedly different suggesting that they have distinct biological roles. Whereas the WFIKKNRP gene is expressed primarily in ovary, testis and brain, the most significant expression of the WFIKKN gene is observed in adult pancreas, liver and thymus.

In view of the presence of WAP-, Kazal-, Kunitz- and NTR-modules (which are frequently involved in inhibition of proteases) in a single multidomain protein we have suggested that these proteins function as multivalent protease inhibitors.

In order to test this hypothesis, in the present work we have expressed the second Kunitz-type protease inhibitor domain of the human protein WFIKKN in *Escherichia coli*. Our structural studies on the recombinant protein have shown that the protein adopts a structure typical of the Kunitz-domain family. The recombinant protein was found to show remarkable specificity for trypsin in contrast to its lack of activity for elastase, chymotrypsin and various proteases with trypsin-like specificity.

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Abbreviations: BPTI, bovine pancreatic trypsin inhibitor; NPGB, *p*-nitrophenyl-*p*-guanidinobenzoate; pNA, *p*-nitroanilide; TFPI, tissue factor pathway inhibitor; WAP, whey acidic protein.

Definition: The nomenclature for the substrate amino acid residues Pn-P4-P3-P2-P1-P′1-P′2-P′3-P'n., where -P1-P′1- denotes the hydrolyzed bond, and Sn-S4-S3-S2-S1-S′1-S′2-S′3-S′4 denote the corresponding enzyme binding sites is described fully in [1].

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Experimental procedures

Restriction enzymes, PCR primers, vectors, bacterial strains

Restriction enzymes were purchased from Promega (Madison, WI, USA) and New England Biolabs (Beverly, MA, USA). The M13 sequencing reagents used for dideoxy sequencing of cloned DNA fragments were from Promega. PCR primers were obtained from Integrated DNA Technologies (Coralville, IA, USA). Plasmid pMed23 was from P. Venetianer (Biological Research Center, Szeged, Hungary). *E. coli* strain JM109 was used to propagate and amplify expression plasmids. The pMed23 expression plasmid contains an ampicillin resistance gene for the selection of the positive clones [4].

Proteases and protease substrates

Bovine trypsin (Sigma-Aldrich, St. Louis, MO, USA), bovine elastase (Serva, Heidelberg, Germany), bovine pancreatic alpha-chymotrypsin (Worthington, Lakewood, NJ, USA), bovine thrombin, human plasmin, human lung tryptase, human high molecular mass urokinase, human tissue plasminogen activator, human plasma kallikrein and porcine pancreatic kallikrein (Calbiochem, Affiliate of Merck, Darmstadt) were commercial preparations.

The synthetic substrates *N*-succinyl-Ala-Ala-Pro-Phe-pNA and *N*-α-benzoyl-L-Arg-pNA (L-BAPNA) were purchased from Sigma, D-Val-Leu-Lys-pNA and D-Pro-Phe-Arg-pNA were from Serva. Glu-Gly-Arg-pNA, D-Ile-Pro-Arg-pNA, Bz-Phe-Val-Arg-pNA, D-Val-Leu-Arg-pNA and succinyl-Ala-Ala-Ala-pNA were obtained

from Bachem (Bubendorf, Switzerland). *p*-Nitrophenyl-*p*-guanidinobenzoate was a product of Fluka (Buch, Switzerland).

Cloning and expression of the second Kunitz-type protease inhibitor module of human WFIKKN protein

On the basis of the known sequence of the human WFIKKN mRNA (GenBank accession number AF422194) we have designed PCR primers for the amplification of the cDNA segment encoding its second Kunitz-domain. The DNA segment coding for the second Kunitz-module of human WFIKKN protein (residues Asp357–Pro412) was amplified with the 5′-GAG TCG ACC GAC GCC TGC GTG CTG CCT GC-3′ sense, and 5′-GCA AGC TTA CGG CAC GGG GCA GGC ATC CTC-3′ antisense primers from a plasmid containing the cDNA coding for WFIKKN protein. The amplified DNA was digested with *Hin*dIII and *Sal*I restriction endonucleases and ligated into M13mp18 Rf digested with the same enzymes. The sequence of the cloned DNA was verified by dideoxy sequencing.

The DNA fragment encoding the second Kunitz-module of WFIKKN was excised from M13mp18 by *Hin*cII/*Hin*dIII digestion and ligated into pMed23 expression vector cut with *Pvu*II/*Hin*dIII. *E. coli* JM109 cells were transformed with the ligation mixture and plated on LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) containing 100 $\mu g \cdot mL^{-1}$ ampicillin.

E. coli JM109 cells carrying the expression vector were grown, and expression of β-galactosidase fusion proteins was induced with 100 μM isopropyl thio-β-D-galactoside. The fusion products were isolated from inclusion bodies by dissolving them in 60 mL of 0.1 M Tris/HCl, 8 M urea, 10 mM EDTA, 0.1 M dithiothreitol (Sigma-Aldrich), pH 8.0. The solution was incubated at 25 °C for 60 min with constant stirring. Insoluble cellular debris were removed by centrifugation and the solubilized proteins were chromatographed on a Sephacryl S-300 column equilibrated with 100 mM Tris/HCl, 8 M urea, 10 mM EDTA, 0.1% 2-mercaptoethanol. The fractions containing the fusion proteins were identified by SDS/PAGE and pooled. The isolated recombinant proteins were refolded by dialysis against 100 mM Tris and 10 mM EDTA pH 8.0 buffer, for 24 h, then against 0.1 M ammonium bicarbonate pH 8.0 buffer.

The β-galactosidase moiety of the recombinant fusion protein was removed by limited elastase digestion. The recombinant protein (1 $mg \cdot mL^{-1}$) was dissolved in 0.1 M ammonium bicarbonate buffer and incubated with 10 $\mu g \cdot mL^{-1}$ elastase (Serva) at 25 °C for 60 min. The reaction was arrested with 2 mM phenylmethanesulfonyl fluoride (Serva) and the protein was lyophilized. The digested recombinant protein was separated from the β-galactosidase fragment on Sephadex G-50 column, equilibrated with 0.1 M ammonium bicarbonate, pH 8.0. Fractions containing the Kunitz-module were pooled, and lyophilized.

The protein was further purified by trypsin-Sepharose affinity chromatography according to described procedures [5,6]. The protein was dissolved in 50 mM Tris-HCl pH 7.5 and applied on a 5-mL trypsin-Sepharose column. The column was washed with four volumes of 50 mM Tris-HCl pH 7.5 and the bound protein was eluted with 100 mM glycine/HCl buffer, pH 2.0. The pH of the eluted fraction was adjusted to 8.0, the protein was desalted on a G-25 Sephadex column equilibrated with 0.1 M ammonium bicarbonate pH 8.0 buffer, and lyophilized.

Sequence analysis of the purified protein with a PE-Applied Biosystems Ltd Procise protein sequencing system showed that the elastase cleavage occurred at the boundary of the β-galactosidase region of the β-gal fusion protein. The amino acid sequence of the resulting purified protein was RT**DACVLPAVQGPCRGWEPRWAYS PLLQQCHPFVYGGCEGNGNNFHSRESCEDACPVP**, where the residues corresponding to the second Kunitz domain of human WFIKKN are in bold. The N-terminal residues RT are part of the vector construct.

Protein analyses

The composition of protein samples was analysed by tricine/SDS/PAGE using 16% slab gels under both reducing and nonreducing conditions [7]. The gels were stained with Coomassie brilliant Blue G-250. The concentration of the recombinant Kunitz-module was determined using the extinction coefficient 14300 $M^{-1} \cdot cm^{-1}$. The extinction coefficient was determined by using the online protein analysis tool, PROTPARAM (http://us.expasy.org/tools/protparam.html).

Circular dichroism spectroscopy

CD spectra were measured over the range of 190–250 nm by using a JASCO J-720 spectropolarimeter thermostatted with a Neslab RT-111 water bath. The measurements were carried out in 1 mm pathlength cells and protein solutions of approximately 0.1 $mg \cdot mL^{-1}$ in 10 mM Tris/HCl, pH 8.0 buffer. All spectra were measured at 25 °C with a 8-s time constant and a scan rate of 10 $nm \cdot min^{-1}$. The spectral slit width was 1.0 nm. All measurements represent the computer average of three scans. Secondary structure of the recombinant protein was estimated from the CD spectra with the CDPRO software (http://lamar.ColoState.EDU/~sreeram/CDPro/index.html [8–10]). Thermal unfolding of the protein was monitored at 203 nm at a heating rate of 60 $°C \cdot h^{-1}$.

Effect of the recombinant protein on the activity of proteases

The activity of the proteases on synthetic peptide-pNA substrates was monitored spectrophotometrically using a Carry 300 Scan spectrophotometer. Hydrolysis of peptide-pNA conjugates was monitored at 410 nm and the initial rates of the reaction were determined.

In the case of bovine trypsin, stock solutions were prepared in 1 mM HCl, 20 mM $CaCl_2$, the active site concentration of trypsin was determined by titration with NPGB according to a described procedure [11]. Stock solutions of the Kunitz-module were prepared in 25 mM Tris, 5 mM $CaCl_2$ pH 7.5 buffer.

The kinetic parameters of trypsin-catalysed hydrolysis of Bz-Phe-Val-Arg-pNA were determined by incubating trypsin (30 nM final concentration) in 25 mM Tris, 5 mM $CaCl_2$, pH 7.5 for 5 min at 37 °C, after which Bz-Phe-Val-Arg-pNA (100–400 μM final concentration) was added and

the enzymatic formation of pNA was monitored at 410 nm, employing a $\Delta\varepsilon$ of 8800 $M^{-1}\cdot cm^{-1}$.

The value of the equilibrium constant for the inhibition of trypsin by the Kunitz-module was determined by measuring its inhibitory effect on the enzymatic hydrolysis of Bz-Phe-Val-Arg-pNA substrate at 37 °C. Aliquots of 250 μL assay mixtures containing 30 nM enzyme and 15, 30, 60 and 150 nM inhibitor were incubated for 5 min at 37 °C in 25 mM Tris, 5 mM $CaCl_2$, pH 7.5 buffer. Bz-Phe-Val-Arg-pNA (100–400 μM final concentration) was then added and the activity was recorded. All experiments were run three times. The enzymatic hydrolysis of the substrate was always corrected for spontaneous hydrolysis.

The dissociation constant of the trypsin–inhibitor complex, K_i was determined from the replot of the apparent K_m values vs. the inhibitor concentration at which they were obtained.

In the case of chymotrypsin, plasmin, thrombin, tissue plasminogen activator and plasma kallikrein, the proteases were preincubated for 30 min at 37 °C in 50 mM Tris, 100 mM NaCl, 2 mM $CaCl_2$, 0.01% Triton X-100 pH 7.5 buffer in the presence of increasing inhibitor concentrations (up to 1 μM final concentration of the inhibitor). The reactions were initiated by adding the appropriate substrate specific for the enzyme. The reaction mixtures contained the following initial enzyme and substrate concentrations: alpha-chymotrypsin was measured at an enzyme concentration of 50 nM and 80 μM *N*-succinyl-Ala-Ala-Pro-Phe-pNA substrate concentration; human plasmin at 10 nM enzyme and 300 μM D-Val-Leu-Lys-pNA substrate concentration; bovine thrombin at 100 nM enzyme and 200 μM Bz-Phe-Val-Arg-pNA substrate concentration; human plasma kallikrein at 3 nM enzyme and 650 μM D-Pro-Phe-Arg-pNA substrate concentration, and the inhibition of human tissue plasminogen activator was measured at 44 nM enzyme and 100 μM D-Ile-Pro-Arg-pNA concentration.

In the case of elastase, pancreatic kallikrein, lung tryptase, urokinase activity was monitored following preincubation of the protease with inhibitor (up to 1 μM final concentration of the inhibitor) for 30 min at 37 °C in the appropriate buffer (see below). Reactions were initiated with substrate to achieve the following initial component concentrations: bovine elastase in 100 mM Tris, 0.05% Triton X-100, pH 8.0 with $[E_0]$ = 38 nM and 600 μM succinyl-Ala-Ala-Ala-pNA; porcine pancreatic kallikrein in 50 mM Tris, 100 mM NaCl, 2 mM $CaCl_2$, 0.01% Triton X-100 pH 8.4 with $[E_0]$ = 16 $U\cdot mL^{-1}$ and 200 μM D-Val-Leu-Arg-pNA; human lung tryptase in 50 mM Tris, 120 mM NaCl, 44 $\mu g\cdot mL^{-1}$ heparin pH 7.5 with $[E_0]$ = 22 nM and 100 μM *N*-α-Benzoyl-L-Arg-pNA; human urokinase in 50 mM Tris, 10 mM EDTA, 50 mM NaCl, 0.5% Triton X-100 pH 8.0 with $[E_0]$ = 30 nM and 300 μM Glu-Gly-Arg-pNA.

Sequence analyses

The amino acid sequences of human WFIKKN protein (AAL18839), human WFIKKNRP protein (AAL77058), bovine pancreatic trypsin inhibitor (bpt1_bovin, P00974), human bikunin (ambp_human, P02760), human Alzheimer's disease amyloid a4 protein precursor (a4_human, P05067) and human type 1 and type 2 hepatocyte growth factor activator inhibitors (spt1_human, O43278; spt2_human, O43291) were taken from NCBI's protein sequence databases.

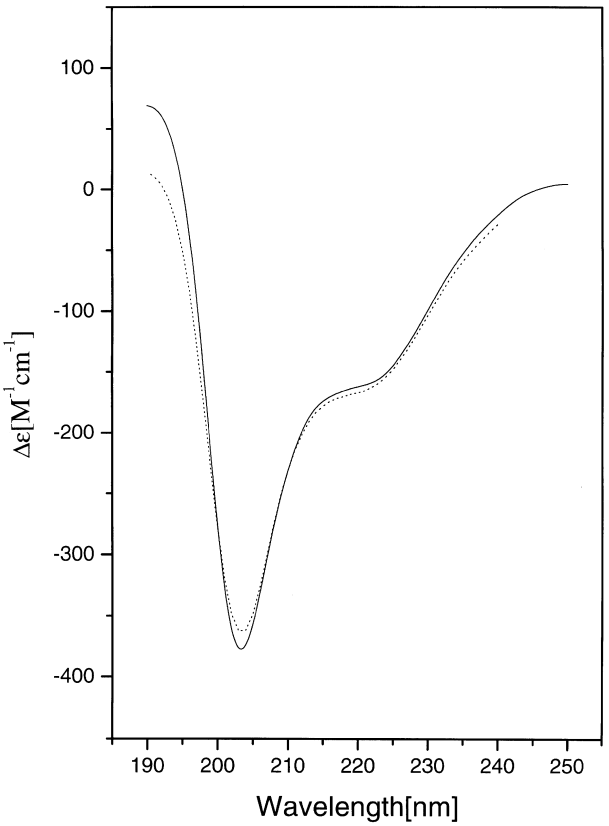

Fig. 1. Far UV circular dichroism spectra of the second Kunitz–type protease inhibitor module of human WFIKKN. The solid line indicates the spectrum of the recombinant protein, the dotted line indicates the CDPro-predicted spectrum of a protein consisting of 0.051 regular β-strand, 0.062 distorted β-strand, 0.110 regular α-helix, 0.183 distorted α-helix, 0.284 turn and 0.309 unordered structure. Spectra were recorded in 10 mM Tris/HCl, pH 8.0 at 25 °C using 0.1 $mg\cdot mL^{-1}$ of protein.

By searching genomic databases of *Fugu rubripes* (http://bahama.jgi-psf.org/fugu/bin/fugu_search; http://www.ncbi.nlm.nih.gov/PMGifs/Genomes/fugu.html; http://fugu.hgmp.mrc.ac.uk/blast/blast.html) with the human WFIKKN and WFIKKNRP sequences as query sequences we have identifed three pufferfish genes/proteins with the same domain organization as human WFIKKN and WFIKKNRP. An ortholog of the human WFIKKN protein (on Scaffold 218), two genes closely related to the human WFIKKNRP protein (WFIKKNRP1 on Scaffold 1054, WFIKKNRP2 on scaffolds 19035 and 2327) were identified in the genome of *F. rubripes*. Using human WFIKKN and WFIKKNRP sequences as query sequences we have identified the C-terminal part (containing only the C-terminal Kunitz- and NTR-domains) of a WFIKKNRP related protein of the Cephalochordate *Branchiostoma belcheri* in NCBI's EST database (AU234635).

Multiple alignments of the amino acid sequences of Kunitz-domains were constructed using CLUSTAL W [12].

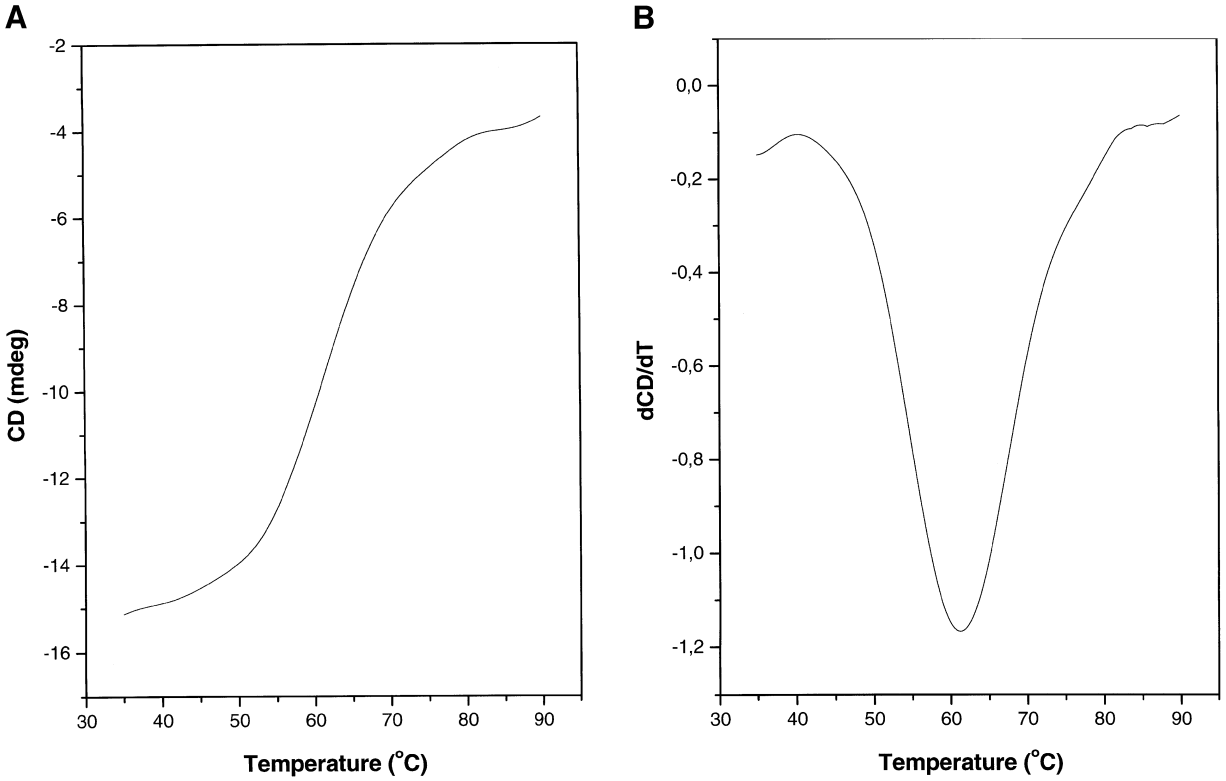

Fig. 2. Temperature dependence of the CD spectra of the second Kunitz–type protease inhibitor module of human WFIKKN protein. (A) Changes in the CD of the protein were monitored at 203 nm in 10 mM Tris/HCl buffer, pH 8.0, during the course of heating from 40 °C to 90 °C at a heating rate of 60 °C·h^{-1}. (B) Melting temperature was determined by derivative processing of changes in CD (cf. part A) using the J-700 STANDARD ANALYSIS program for WINDOWS, v1.30.00.7 (JASCO).

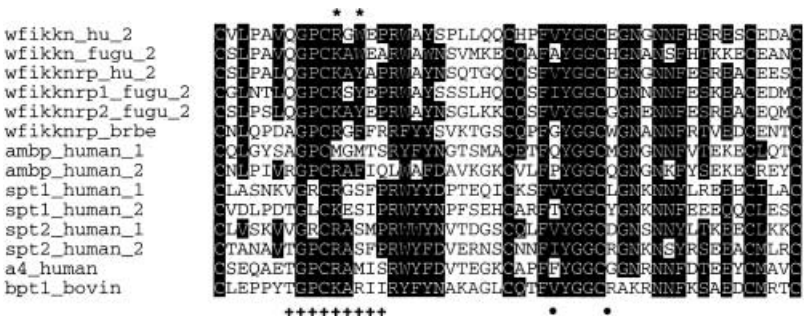

Fig. 3. Alignment of the sequences of the second Kunitz-modules of the human and fugu WFIKKN proteins (wfikkn_hu_2; wfikkn_fugu_2) with the second Kunitz-domains of the human and the two fugu WFIKKNRP proteins (wfikknrp_hu_2; wfikknrp1_fugu_2; wfikknrp2_fugu_2), wfikkn_hu_2), the Kunitz domain of WFIKKNRP of the amphioxus *Branchiostoma belcheri* (WFIKKN_BRABE), and the Kunitz domains of bovine pancreatic trypsin inhibitor (bpt1_bovin), human bikunin (ambp_human_1, ambp_human_2), human Alzheimer's disease amyloid a4 protein precursor (a4_human) and human type 1 and type 2 hepatocyte growth factor activator inhibitors (spt1_human_1, spt1_human_2, spt2_human_1, spt2_human_2). In the bottom line (+) signs mark the P5, P4, P3, P2, P1, P′1, P′2, P′3, P′4 positions, while residues of the secondary sites are indicated by dots. In the top line, asterisks highlight the P1 and P′2 sites. Residues conserved in at least 50% of the aligned sequences are shown by white letters on a black background. Conserved residues are grouped as follows: F,Y,W; I,L,V,M; R,K; D,N; E,Q; T,S.

Results and discussion

Structural characterization of the recombinant Kunitz-module of human WFIKKN protein

The circular dichroism spectra of the second Kunitz-module of WFIKKN protein (hereafter referred to as WFIKKN-KU2) are very similar to those of other members of the Kunitz-domain family [13,14] inasmuch as it is also characterized by a deep trough at 203 nm and a shoulder at 215 nm (cf. Figure 1). Analysis of the spectra with the CDPRO software predicted 5.1% regular β-strand, 6.2% distorted β-strand 11.0% regular α-helix, 18.3% distorted α-helix, 28.4% turn and 30.9% unordered structure.

The presence of both β-strands and α-helices in WFIKKN-KU2 is consistent with the fact that all homologues of WFIKKN-KU2 are known to contain β-strands and α-helices in equivalent positions [14–19]. In view of the fact that the structure of the Kunitz inhibitor of the sea anemone *Stichodactyla helianthus* is nearly identical with that of the bovine pancreatic trypsin inhibitor despite a mere 35% of sequence similarity between the two proteins [16] we can assume that the structure of WFIKKN-KU2 (41% identical with the sequence of BPTI) also has a typical Kunitz-fold.

The thermal unfolding of the recombinant WFIKKN-KU2 protein has been characterized by monitoring changes of CD spectra. As shown in Fig. 2, changes in the CD spectra at 203 nm reflect a single, sharp transition with a T_m value of 61 °C, indicating that the protein collapses in a highly cooperative fashion. It should be noted that the thermal stability of WFIKKN-KU2 is somewhat lower than that of the closely related bovine pancreatic trypsin inhibitor or the chymotrypsin inhibitor of *Bungarus fasciatus* which have been shown to retain most of their native structure at 80 °C [14].

Functional characterization of the second Kunitz-domain of the WFIKKN protein

In view of the fact that an arginine residue is present in the P1 position of WFIKKN-KU2 (Fig. 3), it was not unexpected that the recombinant WFIKKN-KU2 protein did not inhibit the proteolytic action of chymotrypsin or elastase even when tested at 100 μM final concentration. (This observation has permitted the use of elastase to remove the β-galactosidase portion from the refolded fusion protein; see Experimental procedures).

Next, we studied the effect of the WFIKKN-KU2 protein on trypsin and a panel of other serine proteases with specificity for Arg-X or Lys-X peptide bonds. These studies have shown that WFIKKN-KU2 is an efficient inhibitor of trypsin, the dissociation constant for its complex with trypsin (K_i) was 9.6 nM (Fig. 4).

WFIKKN-KU2 was found to display a striking specificity for trypsin. When the inhibitor was employed at 1 μM final concentration, complete inhibition of trypsin 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. Such a marked trypsin-specificity is somewhat unusual among Kunitz-domains. For example, the Kunitz domains of BPTI, amyloid precursor protein, amyloid precursor protein homolog display broader specificity, inasmuch as at 1 μM concentration they inhibit chymotrypsin, glandular kallikrein, plasmin as well as trypsin [5].

We suggest that the explanation for such a marked trypsin specificity of WFIKKN-KU2 lies in the presence of a Trp-residue at the P′2 site of the inhibitor. In the case of Kunitz-domains it is now well established that the primary sites interacting with the target proteases (and determining their protease-specificity) are found in a short segment containing the second conserved cysteine, a secondary site contacting the target proteases includes residues adjacent to the fourth conserved cysteine ([18] cf. Fig. 3). Among all the

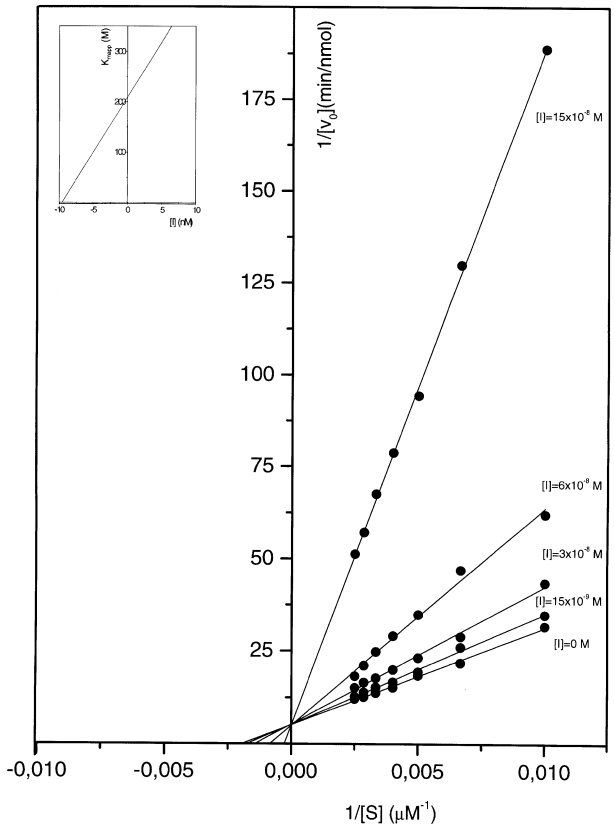

Fig. 4. Lineweaver–Burk plots of the activity of trypsin (30 nM) recorded at different concentrations of the second Kunitz-type protease inhibitor domain of WFIKKN (0, 15, 30, 60 or 150 nM). Hydrolysis of Bz-Phe-Val-Arg-pNA was monitored at 37 °C in 25 mM Tris, 5 mM $CaCl_2$, pH 7.5 buffer. The inhibition constant was calculated by replotting the apparent K_m values (inset).

contact sites, the P1 and the P′2 site play the most critical roles in determining the target specificity of a Kunitz inhibitor [18]. The P1 site interacts with the S1 binding pocket (residues 189–195, 214–220 of target proteases), the P′2 site interacts with the S′2 pocket (residues 151, 192–193 of the target proteases).

As shown in Fig. 3., the putative functional sites determining the target-specificity of the WFIKKN-KU2 domain are quite similar to the corresponding segments of other Kunitz-domains, with one major exception: a Trp residue is found in the P′2 position. A survey of the sequences of Kunitz domains deposited in public databases has revealed that the WFIKKN-KU2 domain and its pufferfish ortholog are unique in that they are the only ones which have a bulky Trp residue at this position.

A key determinant of the hydrophobic S′2 binding pocket of trypsins is the side-chain of Tyr151 [18]. The importance of this residue is underlined by the fact that in the case of the second Kunitz-domain of TFPI its complex with trypsin is stabilized by favorable stacking interaction of Tyr17 (the P′2 residue of the inhibitor) with the Tyr151 side-chain of trypsin [17]. It seems probable that the aromatic Trp residue at the P′2 position of WFIKKN-KU2 also makes favorable contacts with the Tyr151 of

trypsin. It is noteworthy in this respect that the majority of the proteases tested in the present study have nonaromatic residues in positions equivalent to Tyr151 of trypsin (Thr in bovine chymotrypsin, Leu in bovine elastase, Ile in human plasma kallikrein, Gly in human plasmin, Gln in human thrombin, Pro in human lung tryptase), raising the possibility that the inability of WFIKKN-KU2 to inhibit these proteases is partly due to the lack of such a favorable interaction of the P′2 Trp with the target enzymes. The fact that the Trp residue at the P′2 position of WFIKKN-KU2 is conserved from pufferfish to human (cf. Fig. 3) is consistent with the notion that this residue has a major functional importance.

In view of the marked trypsin-specificity of WFIKKN-KU2 it seems plausible to assume that its physiological function is to inhibit trypsin. It should be pointed out, however, that the affinity of WFIKKN-KU2 toward pancreatic trypsin ($K_i = 9.6 \times 10^{-9}$ M) is somewhat weaker than that observed for many other Kunitz inhibitors for their specific target proteases. For example, the Kunitz-domains of placental bikunin (hepatocyte growth factor activator inhibitor type 2) inhibit their target proteases (plasmin, plasma kallikrein) with K_i values in the 10^{-9}–10^{-10} M range [20], the second Kunitz-domain of tissue factor pathway inhibitor inhibits factor Xa with a K_i value of 1.5×10^{-10} M [17].

The relatively high K_i value of isolated WFIKKN-KU2 domain towards pancreatic trypsin raises the possibility that its primary physiological target may be a trypsin-like protease distinct from pancreatic trypsin. Nevertheless, it is likely that the trypsin inhibitory activity of WFIKKN-KU2 has physiological relevance. First, the affinity of the second Kunitz-domain for trypsin may be higher in the case of the intact WFIKKN protein than that of the isolated WFIKKN-KU2 domain. Second, for an inhibitor to be physiologically efficient only its local concentration has to be higher than its K_i value. Our observation that the WFIKKN gene is expressed primarily in the pancreas [2,3] suggests that the local concentration of the WFIKKN protein in this organ may reach levels high enough to control pancreatic trypsin activity.

The biological role of the WFIKKN protein is not limited to the pancreas. We have shown previously that in addition to pancreas, the protein is also expressed in liver, lung and kidney [2,3]. The fact that human trypsins 1, 2 and 3 are also expressed in liver, lung and kidney [21] is consistent with the notion that the WFIKKN protein may also serve as a trypsin inhibitor in these tissues.

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