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Cloning, expression and characterization of human kininogen domain 3

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The internal domain 3 of the heavy chain of human kininogen, a cysteine proteinase inhibitor, was amplified by a polymerase chain reaction from the kininogen cDNA clone phKG36. The DNA fragment was expressed in *Escherichia coli* using the ompA expression vector pASK40 and the resulting protein was isolated from periplasm, purified by *S*-carboxymethylpapain affinity- and ion-exchange chromatography. The recombinant human kininogen domain 3 is 92% pure, reacts with anti-kininogen antibodies and is actively inhibitory. The expected amino acid sequence of ANSM-[G253-S377] kininogen was confirmed; the inhibitor has a molecular mass of 14,396 Da and an isoelectric point of 6.0 (pH). The determined K_i values of the complexes with papain and cathepsin L are similar to those measured previously with proteolytically liberated kininogen domain 3, and those of single-domain cystatins, like chicken egg white cystatin. However, recombinant kininogen domain 3 is a weak inhibitor of cathepsin B (K_i = 63 nM) as it has been found for native L-kininogen (K_i = 340 nM).

Kininogen; Human; Cysteine proteinase inhibitor; Cystatin; Periplasmic expression; *E coli*

1. INTRODUCTION

The kininogens are well studied multifunctional glycoproteins [1–3]. In human plasma two forms are present, H-kininogen (high molecular weight or HK) and L-kininogen (low molecular weight or LK). From both kininogen species kinins can be liberated by limited proteolysis by kallikreins [4]. Kininogens are known to be involved in the inflammatory process, including activation of the intrinsic clotting cascade. More recently it was shown that they may also regulate the activity of cysteine proteinases such as cathepsin B, H and L, as well as calpain I and II [5–9].

H-Kininogen and L-kininogen are single-chain plasma glycoproteins each composed of an N-terminal heavy chain, the kinin segment and a C-terminal light chain. The heavy chains and the kinin segments of both kininogens have identical amino acid sequences. This part of the kininogen structure can be subdivided into 4 domains, the cystatin-like domains, D1, D2 and D3, and the kinin segment, D4. The light chains of both kininogens differ remarkably; they can be subdivided into domain D5 (LK) and D5, D6 (HK), respectively [3,10–12].

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Abbreviations: rKD3, recombinant human kininogen domain 3 (ANSM-[G253-S377] kininogen); PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactoside.

In vitro different cleavage products of kininogens were liberated by limited proteolysis and analysed [12,13]. Salvesen et al. [12] showed that domains D2 and D3 are potent inhibitors of papain and cathepsin L, domain D2 is also an inhibitor of chicken calpain, whereas domain D1 is not actively inhibitory against any of the proteinases tested so far. Recent studies of Schmaier and co-workers with fragments produced by proteolytic cleavage in vitro indicate that domain D3 contains a cell binding region for platelets, and, in addition, another region which inhibits thrombin binding and thus activation of platelets [14].

Detailed structural and functional studies with single domains of the kininogens are limited by the quantity, purity and structural homogeneity of the available starting material. Hence, defined recombinant kininogen domains would offer an interesting alternative as compared to fragments liberated from natural kininogens, which are frequently truncated, or to synthetic peptide fragments of the domains. Furthermore, variants of recombinant domains can be used to analyze structure–function relationships.

Here we report the design, cloning, expression, purification and biochemical characterization of a recombinant unglycosylated kininogen domain 3 (rKD3).

2. MATERIALS AND METHODS

2.1. *Materials and general methods*

Chemical reagents and materials for molecular biology, protein chemistry and biochemistry studies were purchased from Merck, Sigma, Boehringer, New England, Biolabs, Applied Biosystems, Bachem. Oligonucleotide primers were synthesized in the laboratory

of Prof. H. Wolf, Max von Pettenkofer Institut, München, the cDNA clone phKG36 was kindly supplied by S. Nakanishi, Kyoto. The polymerase chain reactions (PCRs) were accomplished using equipment from Perkin-Elmer. The standard techniques of molecular cloning were performed as described elsewhere [15]

2.2. *PCR and molecular cloning*

The *Eco*RI-linearized cDNA clone, pHKG36 [10], was used as a template for PCR with the primers a1: GGGAATTCCA TGGGGAAGGA TTTTGTACAA CCACCTACC containing an *Eco*RI and a *Nco*I site, and b1: GAGGATCCGT CGACTTACTA TGAGATCATT CCCAGTGGTT GACA containing a *Bam*HI, a *Sal*I site and two stop codons. The PCR was carried out according to a standard procedure [16]. The fragments were precipitated with ethanol, digested with *Eco*RI and *Sal*I, purified on a low melting-point agarose gel (2% NuSieve, SeaKem) and isolated using the kit of Genclean (Genclean; Bio 101). About 60 fmol of the expression unit were ligated with 6 fmol of the appropriately prepared pASK40 DNA [17]. Competent *E coli* JM 83 cells (ara,Δ(lac-proAB),rpsL,phi80, lacZΔM15) were transformed. Transformants were selected after restriction analysis and DNA sequencing (Pharmacia, T7 sequencing kit) of plasmid DNA. Expression was analysed after SDS/PAGE of total *E coli* proteins.

2 3. *Expression, fermentation and isolation*

The selected *E coli* strain 1263 (harbouring the vector pDR13.1 1) was grown at 33°C in 2 × YT media (200 μg ampicillin/ml), induced with 1 mM IPTG at OD_{550} of approx. 0.8 for about 5–7 h either in shaker flasks or in a BioStat E fermenter with an ES-10 culture vessel (Braun-Diessel Biotech) at 20°C, 550 rpm, pH 7.4, aeration 5 standard l/min and foam control. Cells were harvested by centrifugation (Sorvall RC3B, 5000 rpm, 15 min at 8°C) and the recombinant protein was isolated from periplasma [18]. Cells were resuspended at 0°C for 4 min in 20% w/v sucrose, 0.2 M Tris-HCl, pH 8.0, 0.1 M EDTA, centrifuged at 6,000 × *g* and the supernatant was filtrated through a Filtron minisette omega 0.16 μm membrane. The recombinant material of this periplasmic fraction was purified by *S*-carboxymethylpapain affinity chromatography [18] followed by ion-exchange chromatography on Fractogel EMD-TMAE 650S, Merck, loaded with 25 mM bisTris-HCl, pH 6.0 and eluted with a pH gradient from 6 0 to 4.8 [20] on a BioPilot system from Pharmacia.

2.4. *Protein characterisation*

The amount of protein was quantified by A_{280} determination using A_{280} 1% = 1 ng/nl and by the Bradford assay from Bio-Rad

SDS-PAGE was performed on 10–20% polyacrylamide gels according to Laemmli [21] and gels were stained with Coomassie blue or silver Polyclonal and monoclonal antibodies (anti-kininogen IgG, AS 165 and HKH 11, kindly provided by W. Muller-Esterl, Mainz) were used for immuno printing after Western blot analysis [22]. Purified proteins (2–3 nmol) and tryptic digests of them were resolved by HPLC on a reverse-phase column (100 RP-18, 5 μm Licrochart, Merck) as described for AEF [S1 M M29I M89L] chicken cystatin [19]. Amino acid sequence analyis was done with a gas-phase sequencer 473 A (Applied Biosytems) following the instructions of the manufacturer.

2.5. *Inhibiton assay and determination of kinetic constants*

The papain inhibitory activity of the material was determined according to Barrett and Kirschke [23]. Kinetic constants for the interaction of rKD3 and human L-kininogen (Medor) with papain, actindin, human cathepsin B and human cathepsin L, were determined using materials and methods as described [19,24,25].

3. RESULTS AND DISCUSSION

3.1. *Design and cloning of rKD3*

In order to produce a biosynthetically stable and functionally active protein domain from a cDNA of a multifunctional protein the appropriate segment has to be carefully selected. For the construction of a cDNA expression unit of the human kininogen domain D3 we considered the results available on limited proteolysis of kininogens [12,13], sequence alignment of cystatins [26], the exon–intron junctions of kininogen genes [11], and our experience in synthesis and expression of recombinant cystatin variants. Using primers a1 and b1, together with the cDNA phKG36, a DNA fragment was produced by PCR. The material was purified and ligated into pASK 40, a periplasmatic *E. coli* expression vector. The expected DNA sequence coding for ANSM

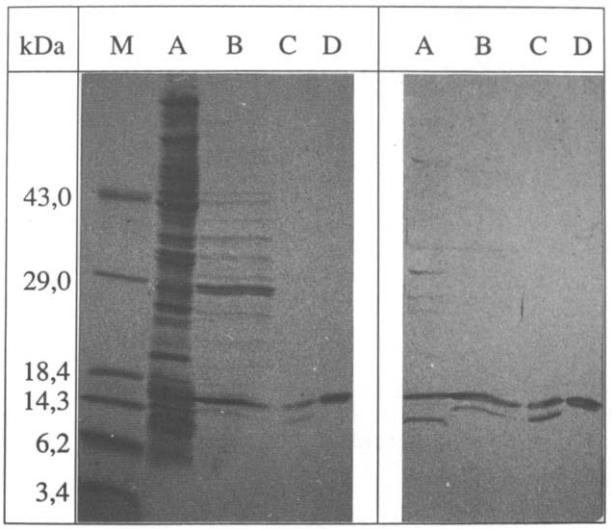

Fig. 1. SDS-PAGE and corresponding immunoblot analysis (monoclonal antibody HKH11 directed against domain3 of HK) of *E coli* proteins and isolation products. Lanes: M, low molecular mass marker (indicated in kDa); A, lysate of 6 h induced *E coli* (2×10^8 cells); B, periplasmic fraction of 6 h induced *E. coli* (3 μg); C, eluate from CM-papain Sepharose 4B (2 μg) with ANSM [G253-S377] kininogen domain 3 (upper band) and truncated forms (lower bands), D, main fractions after anion-exchange chromatography (2 μg)

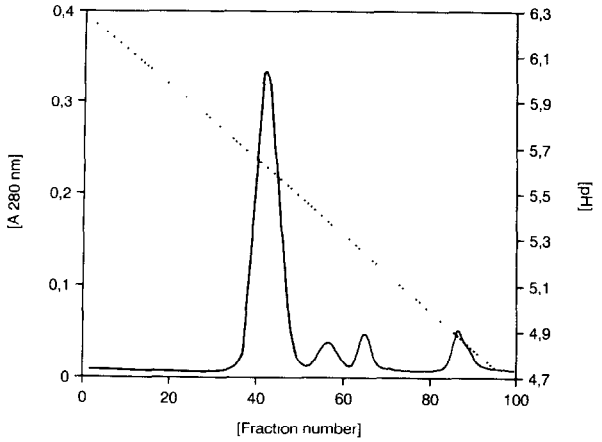

Fig. 2 Elution profile of rKD3 from Fractogel EMD-TMAE anion-exchange chromatography, dotted lines, pH gradient (main fractions from number 38 to 48).

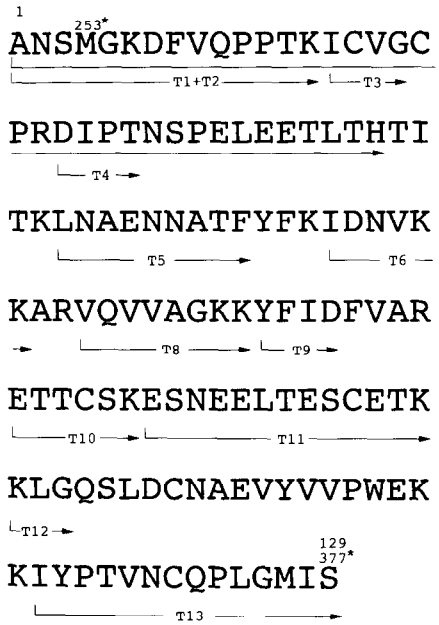

Fig. 3. Amino acid sequence of ANSM [G253-S377] kininogen. The sequenced N-terminal part and the sequenced regions of tryptic peptides (T) are underlined. Numbering with asteriks (*) is according to the amino acid sequence in pHKG36 cDNA clone.

[G253-S377] kininogen was confirmed. The resulting vector was named pDR 13.1.1. and used for transformation of *E. coli* JM 83.

3.2. *Expression and isolation*

The host strain, *E. coli* 1263 (JM 83 × pDR13.1.1.) expressed 2–4 mg rKD3 per l, which is equivalent to 1–2% of total *E. coli* protein as judged by SDS-PAGE (Fig. 1). Using a standard procedure for isolation of periplasmic proteins [17], the actively inhibitory material was found mainly in the buffer solution containing sucrose/EDTA before osmotic-shock treatment. The material was enriched by a factor of approx. 10 after this step (Fig. 1); contamination by other proteins is very low.

Recombinant chicken egg white cystatin, a molecule quite similar to rKD3, was expressed in a 2-fold higher yield [19] and could be obtained mainly in the supernatant after the osmotic-shock treatment. The kininogen domain D2, which is very similar to rKD3, was also constructed and cloned in parallel, but could not be expressed using the approaches for rKD3 and other cystatins [20]. At present we do not know the reason for this different expression and secretion behaviour of quite similar and functionally related molecules in *E. coli*.

3.3. *Purification and protein analysis*

The periplasmatic material was bound to a carboxymethyl-papain affinity column, developed with a pH shift from 6.5 to 11, and the eluant neutralized. The material thus obtained was about 80% pure. The major protein band migrated at the expected position of 15 kDa and showed a specific papain inhibitory activity of 10–40%. It was contaminated with minor additional protein fractions which were identified by reverse-phase HPLC analysis and isoelectric focussing (2 major and 3–4 minor bands with isoelectric points between 5.2 and 6.0 (data not shown)). N-Terminal amino acid sequence analysis revealed four distinct sequences; the expected sequence rKD3 (about 80%) as well as three minor sequences CVGC (12%), MGK (4%) and FVQ (3%), which represent N-terminally truncated forms of rKD3.

Using Fractactiveogel TMAE anion-exchange chromatography we could separate some of the truncated proteins (Fig. 2). The main fraction (Fig. 2) migrated as a single band in SDS-PAGE (Fig. 1), eluted as a sharp peak after HPLC and showed a single isoelectric point of 6.0 (data not shown). However, by N-terminal sequencing we found, as well as the correct rKD3 sequence (91%), three minor N-terminally shortened forms which could not be separated by ion-exchange chromatography. The results of a typical purification of rKD3 from *E. coli* are given in Table I. After immuno printing and Western blotting minor heterogeneous rKD3 expression products could be detected in *E. coli* lysates (data not shown). This may be explained by an inaccurate endoproteolytic cleavage by the signal peptidase which, however, has not been described for

Table I

Results of a typical purification of ANSM[G253-S377] kininogen from *E. coli*

Purification step	Volume	Total protein	Active material	Specific activity	Yield
	(ml)	(mg)	(mg)	(%)	(%)
E. coli cell lysate	1,000	300	n.d	n.d.	
Periplasmic fraction	200	12	1.2	10	100
Cm-papain Sepharose 4B	7	2	0.8	40	66
Fractogel TMAE main fractions	5	0.7	0.3	42	25

Total protein was estimated with the Bradford assay (bovine serum albumin as standard); active material was calculated from papain inhibitory assays; yield is given as percentage of isolated active material; n.d. = not determined.

Table II

Inhibition constants (K_i) and rate constants (k_{on} and k_{off}) for the inhibition of cysteine proteinases by recombinant human kininogen domain 3

Inhibitor	Cathepsin L			Papain			Actinidin	Cathepsin B
	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_{ic} (nM)	k_{on} ($M^{-1} \cdot s^{-1}$)	k_{off} (s^{-1})	K_{ic} (nM)	K_i (nM)	K_i (nM)
ANSM-rKD3	10.5×10^7	1.0×10^{-4}	0.0010	3.0×10^7	5.4×10^{-5}	0.0018	6	63.4
L-Kininogen	3.0×10^7	1.1×10^{-4}	0.0035	2.0×10^7	6.4×10^{-5}	0.0032	23	340
cew cystatin	11.0×10^7	3.5×10^{-5}	0.0003	1.4×10^7	2.0×10^{-5}	0.0014	18	2.9

The corresponding data obtained with L-kininogen (determined in the same set of experiments) and natural chicken egg white cystatin (cew cystatin, from [24] are included for comparison. K_i of the complexes with cathepsin L and papain were calculated from the rate constants as $K_{ic} = k_{off}/k_{on}$; K_i for the interaction with actidinin and cathepsin B were determined in equilibrium inhibition experiments.

ompA up to now but has been shown for pho A expression systems or by other proteolytic enzymes present in the periplasm [27].

To confirm the primary structure of recombinant ANSM [G253-S377] kininogen, 13 out of 17 theoretically expected tryptic peptides were isolated and identified by partial sequencing. The first 36 amino acid residues of the intact protein were also shown by sequence analysis to be identical with the expected sequence. The internal peptides T8, T10, T11, and the C-terminal T13 peptide, were sequenced completely (Fig. 3). As expected, the recombinant *E. coli* protein is not glycosylated.

3.4. *Characterization of inhibitory activity*

Inhibitory activity measured by the papain inhibition assay was detected during all steps of the isolation procedure. The purified material contained about 40% actively inhibitory material if referred to protein concentration (Table I).

The complex dissociation constants (K_i) given in Table II show that rKD3 is a tight-binding inhibitor of papain and cathepsin L but a weak inhibitor of actinidin and cathepsin B. The inhibitory profile of rKD3 resembles that of chicken egg white cystatin with the exception of the 20-fold lower inhibition of cathepsin B by the kininogen domain. The inhibitory effect of rKD3 on papain and cathepsin L is comparable to that of proteolytic fragments of natural L-kininogen comprising domain 3 which have been described earlier [12,13]. Previously reported [13] higher K_i values (8–10 pM for the papain-domain 3 complex) may be explained by the fact that equilibrium had not been achieved in those experiments. The weak inhibition of cathepsin B (K_i = 340 nM) is a characteristic feature of L-kininogen [28]. Until now no quantitative data had been available on the inhibition of cathepsin B by individual kininogen domains. Our results with rKD3 indicate that domain 3 itself has a low affinity for cathepsin B (K_i = 63 nM). The K_i of 340 nM for the inhibition of cathepsin B by native L-kininogen (based on the actively inhibitory concentration obtained by titration with papain) suggests that the affinity of domain 2 for cathepsin B could be even lower than that of domain 3 and might be negligible in the presence of domain 3 under equilibrium conditions. However, as the binding stoichiometry of cathepsin B to human L-kininogen is not precisely known, the observed low affinity of complete L-kininogen for cathepsin B may also be due to mutual steric hindrance of two enzyme molecules on binding to the two adjacent domain.

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