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Enzymatic profiling of human kallikrein 14 using phage-display substrate technology

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Abstract

The human *KLK14* gene is one of the newly identified serine protease genes belonging to the human kallikrein family, which contains 15 members. *KLK14*, like all other members of the human kallikrein family, is predicted to encode for a secreted serine protease already found in various biological fluids. This new kallikrein is mainly expressed in prostate and endocrine tissues, but its function is still unknown. Recent studies have demonstrated that *KLK14* gene expression is up-regulated in prostate and breast cancer tissues, and that higher expression levels correlate with more aggressive tumors. In this work, we used phage-display substrate technology to study the substrate specificity of hK14. A phage-displayed random pentapeptide library with exhaustive diversity was screened with purified recombinant hK14. Highly specific and sensitive substrates were selected from the library. We show that hK14 has dual activity, trypsin- and chymotrypsin-like, with a preference for cleavage after arginine residues. A SwissProt database search with selected sequences identified six potential human protein substrates for hK14. Two of them, laminin α-5 and collagen IV, which are major components of the extracellular matrix, have been demonstrated to be hydrolyzed efficiently by hK14.

Keywords: extracellular matrix protein; kallikrein; phage display; protease; substrate.

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Introduction

The human kallikrein family represents a fascinating protease family in terms of size and physio-pathological interest. One of the best-known members is the human prostatic kallikrein 3, also named prostate-specific antigen (PSA), which is used diagnostically for prostate cancer detection. Very recently, new members of the human kallikrein family were identified, to form the biggest subgroup of human serine proteases with 15 different kallikreins (Yousef and Diamandis, 2001, 2002). The growing interest in kallikreins originates from their correlation with clinicopathological variables of many cancers. Therefore, either for prostate cancer with hK2 (Tremblay et al., 1997; Magklara et al., 2000), hK11 (Nakamura et al., 2003) and hK15 (Stephan et al., 2003), for ovarian cancer with hK5 (Yousef et al., 2003a), hK6 (Diamandis et al., 2003), hK8 (Kishi et al., 2003), and hK10 (Luo et al., 2003) or for breast cancer with hK2, hK3 (Black et al., 2000), hK5 (Yousef et al., 2003a), hK9 (Yousef et al., 2003b), and hK15 (Yousef et al., 2002a), the evaluation of kallikrein levels in blood circulation should offer new tools for cancer detection in the near future (Diamandis and Yousef, 2002).

The human kallikrein 14 gene was identified by two groups (Hooper et al., 2001; Yousef et al., 2001). Yousef et al. (2001) demonstrated by reverse transcriptase-polymerase chain reaction (RT-PCR) that mRNA hK14 expression was predominant in the central nervous system, whereas Hooper et al. (2001) found high expression in prostate and skeletal muscle by *in situ* hybridization and Northern blot analysis. More recently, the development of a specific immunoassay showed that hK14 protein is mainly expressed in breast, skin and prostate tissue (Borgono et al., 2003). This study revealed that hK14 could be a new biomarker for ovarian and breast cancers because serum hK14 levels were increased in 65% and 40% of these cancers, respectively (Borgono et al., 2003). Moreover, hK14 expression was associated with a poor prognosis for breast (Yousef et al., 2002b) and prostate cancers (Yousef et al., 2003c), and was found to be regulated by steroid hormones (Borgono et al., 2003; Yousef et al., 2003d). This led us to hypothesize that the hK14 protease could be involved in cancer biology.

To date, the enzymatic characteristics of hK14 are still unknown. The presence of a highly conserved aspartate residue, six amino acids N-terminal of the serine of the catalytic triad, indicates a trypsin-like activity for hK14 (Hooper et al., 2001; Yousef et al., 2001). Recently, we reported the use of phage-display substrate technology to characterize the enzymatic activity of human kallikrein 2 (Cloutier et al., 2002) and to describe proteases secreted by cancer cells (Cloutier et al., 2004). This method is increasingly used for the characterization of new pro-

teases and the discovery of potential biological targets (Deperthes, 2002). Using this method, we selected highly sensitive substrates and predicted several potential substrates for hK14, two of which were experimentally verified. As expected, the substrate peptides selected indicated that hK14 possesses a trypsin-like activity, but surprisingly, a weak chymotrypsin-like activity as well.

Results

Selection of phage substrate for hK14

The substrate phage library was panned against hK14 to select substrates cleaved by its hydrolytic activity. Cleaved phages were amplified in *E. coli* TG1 cells and then subjected to five more rounds of enzyme digestion and screening. The amount of phages released increased with each round, indicating the presence of a higher number of hK14-susceptible phages after each round of selection. The amino acid sequences of 32 phage peptides from the last round of selection were determined by sequencing. The sequences corresponding to the substrate regions are listed in Table 1. From all peptides selected and cleaved, 69% possess a basic residue in P1 position, as expected for the putative trypsin-like activity of hK14, whereas 31% of peptides have a tyrosine residue specific for chymotrypsin-like activity in P1.

Kinetic characterization of substrate hydrolysis by hK14

To verify that the sequences from the phage display analysis were indeed substrates for hK14, and to identify the cleavage site, all peptides selected were constructed in fluorescent substrate form. Our substrate system is based on the transfer of energy from cyan fluorescent protein (CFP) to yellow fluorescent protein (YFP), which are linked by the substrate. Cleavage of the linker by a protease separates the two fluorophores and results in a loss of energy transfer. Thus, hydrolysis of the substrate can be evaluated by the measurement of increasing fluorescence intensity of the donor at 485 nm, corresponding to the wavelength of CFP emission (Mitra et al., 1996; Felber et al., 2004).

All substrates were hydrolyzed by hK14 with variable levels of efficacy, and k_{cat}/K_m values ranged from 2000 to 481 000 M^{-1} s^{-1}. The specificity of cleavage was demonstrated with CFP-GGGGG-YFP, which is not hydrolyzed by hK14 (data not shown).

These results clearly indicate that the preferred P1 amino acid for hK14 susceptibility is Arg (Table 1) since

Table 1 Comparison of specificity constant (k_{cat}/K_m) values for CFP-X5-YFP substrates based on selected substrates for hK14.

Clone	Sequence									k_{cat}/K_m (M^{-1} s^{-1})
	P5	P4	P3	P2	P1	P1′	P2′	P3′	P4′	
G1	V	G	S	L	**R**					481 000
C11					**R**	Q	T	N	D	415 000
E5			N	Q	**R**	S	S			388 000
E8			L	Q	**R**	A	I			367 000
F11			Q	R	**L**	R	D			307 000
F3			P	D	**R**	H	M			243 000
E2	L	S	G	G	**R**					207 000
G9		T	V	D	**Y**	A				134 000
E7			L	S	**R**	D	N			127 000
D9					**R**	G	K	T	N	80 000
E9	N	N	K	L	**R**					74 000
E6		M	Q	V	**K**	H				34 000
E4	T	T	D	L	**R**					27 000
E12					**R**	V	T	S	T	26 000
E1				A	**Y**	G	Y	K		24 000
G3			S	T	**K**	G	I			20 000
F5			K	L	**K**	E	T			19 000
F6		V	G	L	**Y**	D				18 000
E10		V	V	M	**K**	D				15 000
D11					**R**	V	D	T	G	15 000
F7			G	H	**R**	I	N			12 000
F10					**Y**	Q	S	L	N	12 000
C5	S	D	K	V	**Y**					9000
G11	H	E	T	L	**K**					9000
D7			T	S	**Y**	L	N			9000
F4	M	Q	A	T	**K**					8000
G7	E	A	P	A	**K**					8000
F12	P	V	H	L	**Y**					7000
F1	Q	P	N	G	**Y**					6000
G5				A	**Y**	G	L	A		6000
C9					**Y**	Q	N	S	S	6000
E11		S	A	V	**R**	P				5000

P1 positions of scissile bonds are indicated in bold font.

Table 2 Specificity of phage-selected hK14 substrates toward different human proteases.

Peptide	Sequence	Specificity k_{cat}/K_m (M^{-1} s^{-1})							
		hK14	Trypsin	Chymotrypsin	Elastase	Plasma kallikrein	hK1	HK2	PSA
Trypsin-like substrate									
G1	VGSLR	481 000	270 000	145 000	–	–	–	21 000	–
C11	RQTND	415 000	260 000	251 000	–	–	–	23 000	–
E5	NQRSS	388 000	2 070 000	–	–	5000	–	14 000	–
E8	LQRAI	367 000	2 270 000	–	209 000	5000	–	25 000	–
F11	QRLRD	307 000	1 420 000	168 000	–	–	LC	32 000	–
F3	PDRHM	243 000	319 000	192 000	–	–	–	–	–
E2	LSGGR	207 000	4 676 000	83 000	–	–	–	14 000	–
E7	LSRDN	127 000	246 000	155 000	–	–	–	16 000	–
D9	RGKTN	80 000	2 111 000	94 000	–	–	–	21 000	–
E9	NNKLR	74 000	384 000	77 000	–	–	–	12 000	–
E12	RVTST	26 000	–	100 000	200 000	–	–	–	–
E10	VVMKD	15 000	–	–	65 000	–	–	–	–
Chymotrypsin-like substrate							–		
G9	TVDYA	134 000	–	145 000	181 000	–	–	–	–
E1	AYGYK	24 000	129 000	618 000	–	–	–	–	–
F6	VGLYD	18 000	–	409 000	–	–	–	–	–
F10	YQSLN	12 000	–	134 000	49 000	–	–	–	LC
D7	TSYLN	9000	–	266 000	90 000	–	–	–	–

LC, low cleavage; k_{cat}/K_m not determined; –, no detectable cleavage.

the best hK14 substrates with k_{cat}/K_m greater than 200 000 M^{-1} s^{-1} possess an Arg in the P1 position. Interestingly, from the four peptides cleaved most efficiently by hK14, two contained a Gln at the P2 position. In contrast, a broad variety of amino acids were found in the P1′ position, demonstrating no significant preference at this position. However, two substrates possess an aspartic acid in the P1′ position and are cleaved relatively efficiently.

On the other hand, all substrates with a Lys at the P1 position were cleaved at a low rate with a k_{cat}/K_m value equal to or below 34 000 M^{-1} s^{-1}. Similarly, the cleavage rate for substrates with a P1 tyrosine was very low, except for one substrate, peptide G9, which had a k_{cat}/K_m value of 134 000 M^{-1} s^{-1}. With the exception of the P1′ position, in which a glycine residue is found in approximately 50% of P1 lysine or tyrosine substrates, no amino acid was recovered more frequently at the other positions. Nevertheless, it has to be stated that the majority of glycine residues found in position P1′ originated from the phage linker region flanking the selected pentapeptide substrates, in which Lys or Tyr residues are found in position 5 of the peptide selected.

Specificity of the best substrates selected

Since many of the substrates selected contained motifs potentially susceptible to cleavage by other proteases, we measured the degree to which hK2, plasma kallikrein, PSA, chymotrypsin, trypsin and elastase could cleave these hK14 substrates (Table 2). Each substrate was tested at an enzyme concentration leading to specific cleavage in the substrate linker and not hydrolyzing the GGGGG control substrate.

Not surprisingly, most of trypsin-like substrates are cleaved by trypsin with a variable efficacy, which was not strictly in correlation with hK14 preferences. For instance, the two pentapeptides VGSLR and RQTND were the best substrates for hK14, but were not very efficiently cleaved by trypsin in comparison to other peptides, such as LSGGR, exhibiting a k_{cat}/K_m value of almost 50 000 000 M^{-1} s^{-1}. In contrast, peptides possessing a Gln in P2 position were excellent substrates for hK14, as well as for trypsin. Only two hK14 substrates with low trypsin-like hK14 activity, RVTST and VVMKD, but four out of five substrates with chymotrypsin-like hK14 activity were not cleaved by trypsin.

All chymotrypsin-like substrates were cleaved by chymotrypsin more efficiently than by hK14, except for the substrate TVDYA, which gave almost the same k_{cat}/K_m value with hK14, chymotrypsin and elastase. Elastase also proteolyzed the two peptides TSYLN and YQSLN, which was also cleaved weakly by PSA.

The substrates selected displayed high selectivity for hK14 in comparison to other human kallikreins, such as hK1, hK2, PSA and PK. Only hK2 proteolyzed most of the trypsin-like substrates, with k_{cat}/K_m values always at least five-fold lower than for hK14. For example, NQRSS peptide was 27- and 78-fold more selective for hK14 than for hK2 and PK, respectively, and F3 peptide demonstrated high hK14 specificity and no cleavage with any other kallikrein could be detected.

Identification of potential biological targets

A search in the SwissProt database, using only the best substrates as query sequences, was carried out to retrieve potential natural substrate proteins for hK14. This search identified different potential substrates, presented in Table 3. Interestingly, several components of the extracellular matrix were identified as potential targets. The best substrate sequence, peptide G1, is present in matrilin-4, which is the most recently identified member of the matrilin family of von Willebrand factor A-like domain containing extracellular matrix adapter proteins. Another extracellular matrix protein, the laminin α-5 chain, pos-

Table 3 Identification of potential physiological substrates of hK14 using the SwissProt database.

Selected peptides[a]	Potential protein substrate[b]
E7 (**LSRDN**)	Laminin α-5 chain precursor (residues 2419–2423)
C11 (**RQTN**D)	Serine protease inhibitor Kazal-type 5 precursor (residues 1034–1037) Laminin α-1 chain precursor (residues 1988–1991)
G1 (**VGSLR**)	Matrilin-4 precursor (major component of ECM of cartilage) (residues 178 –182) Villin-like protein (possible tumor suppressor) (residues 61–65)
F11 (**QRLRD**)	Endothelin-2 precursor (vasoconstrictor peptide) (residues 145–149) Vascular endothelial growth factor receptor 3 precursor (residues 1126–1130)
D9 (**RGKT**N)	Collagen α1(XII) chain precursor and (XIX) (residues 1838–1841) Collagen α3 (IV) chain precursor (residues 832–835)
E4 (**TTDLR**)	Proprotein convertase subtilisin/kexin type 5 Precursor (residues 371–375)
E12 (**RVTST**)	Airway trypsin-like protease precursor (residues 6–10)

[a]Amino acid residues within selected substrate sequences identical to potential protein substrates are indicated in bold font.
[b]The position of the homologous sequence within the potential protein substrate is given in parentheses.

sesses a sequence identical to peptide E7 in its C-terminal part and the sequence containing the first four amino acid residues selected in peptide D9 were found in collagen IV and XII. In addition, the searches identified several potential cleavage sites identical to peptides F11 and E4 in the precursor of vasoconstrictor peptide endothelin-2 and the preproprotein convertase subtilisin. An amino acid pattern similar to peptide C11 was found in domain 15 of the serine protease inhibitor Kazal-type 5, which contains a sequence identical to amino acids 1–4 of the selected substrate.

Proteolysis of extracellular matrix proteins

To investigate the proteolytic cleavage of the potential substrates identified, a low amount of hK14 was incubated with either laminin 10/11 or collagen IV. No degradation of BSA was observed under the conditions of hydrolysis, whereas extracellular matrix proteins were degraded very efficiently. Electrophoretic separation under reducing conditions showed that a band of laminin 10/11, corresponding to the α-5chain, was cleaved by hK14, while the other chains remained intact (Figure 1A). Interestingly, after hK14 incubation a fragment of approximately 260 kDa appeared, which corresponds to the expected molecular size of the N-terminal laminin fragment after cleavage at $LSR_{2421}DN$. Analysis under reducing conditions of collagen IV before degradation showed a large smear in the upper part of the gel corresponding to proteins of high molecular weight (Figure 1B). These proteins disappeared under hK14 activity and several major bands between 150 and 220 kDa in size appeared, demonstrating efficient proteolytic hK14 activity toward collagen IV.

Discussion

It has been proposed that human kallikrein 14 is associated with different cancers, such as ovarian, breast (Borgono et al., 2003; Yousef et al., 2002b, 2003d) and prostate cancer (Yousef et al., 2003c). To gain further insights into the substrate specificity of this enzyme, we screened a phage-displayed random library. This study identified two classes of pentapeptide substrates for hK14: trypsin-like and chymotrypsin-like substrates. However, we showed that hK14 has trypsin- rather than chymotrypsin-like cleavage specificity despite the selection of several aromatic residue-containing substrates. The substrates with the highest k_{cat}/K_m values have an arginine in the P1 position, indicating a preference for this amino acid, as was shown for hK1 (Chagas et al., 1991), hK2 (Frenette et al., 1997a; Cloutier et al., 2002), and hK6 (Magklara et al., 2003). Lysine, on the other hand, seems to be less suitable than tyrosine in the P1 position. If the two amino acids were present in the same peptide, hK14 cleaved after the tyrosine residue. In addition, one of the chymotrypsin-like substrates, TVDYA, gave a significantly higher kinetic value, 134 000 M^{-1} s^{-1}, than all the lysine-P1 substrates, with k_{cat}/K_m values not higher than 34 000 M^{-1} s^{-1}. No selectivity of hK14 was observed for the P1′ position, in which different types of amino acids, such as small and uncharged, hydrophobic, positively charged or negatively charged residues have been recovered in the best substrates. In contrast to hK1 and hK2,

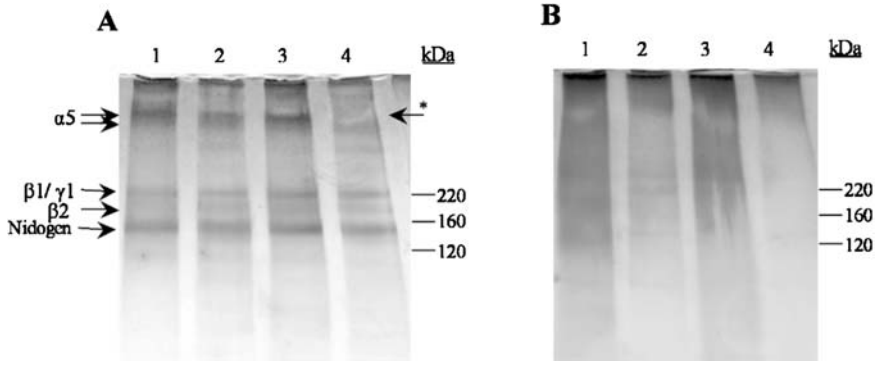

Figure 1 SDS-PAGE analysis of (A) laminin 10/11 and (B) collagen IV after treatment with 200 nM hK14.
Proteins were separated on 4–15% gradient gels under reducing conditions and visualized using silver staining. Laminin 10/11 or collagen IV for 3 h alone (1) or with hK14 (2); laminin 10/11 or collagen IV for 12 h alone (3) or with hK14 (4).

the P1′ position seems to be less important for the kinetics of hK14 hydrolysis. Indeed, hK1 and hK2 showed a high preference for serine residues in P1′, even if they can accommodate a broad range of amino acids, except for basic residues, in this position (Bourgeois et al., 1997; Del Nery et al., 1999; Cloutier et al., 2002).

Analysis of other surrounding positions demonstrated that hK14 can be accommodated by a large variety of amino acids. This observation does not mean that hK14 has a large spectrum of activities like trypsin or chymotrypsin, but demonstrates an ability to cleave different sequences, depending to the context.

The chymotrypsin-like activity of hK14, even if it is inferior to its trypsin-like activity, represents an interesting discovery. To the best of our knowledge, except for the Phe-Phe link cleaved by hK1 in kallistatin and some derived peptides (Pimenta et al., 1999), this is the first human kallikrein described with dual activity. The conformation of the specificity pocket in hK14 should therefore accommodate both aromatic and basic amino-acid side chains at the substrate P1 position to explain the dual chymotrypsin- and trypsin-like activity of hK14. A non-human kallikrein, rat kallikrein rK9, has already been described as a dual-activity enzyme with a strong preference for basic residues and higher trypsin-like activity (Moreau et al., 1992; Zani et al., 2001). This enzyme, like hK14, is mainly expressed in prostate and is regulated by steroid hormones. However, an important structural difference exists between hK14 and rK9, and also the three first human kallikreins, hK1, 2, and 3, since the kallikrein loop is not present in its entirety in the hK14 sequence (Yousef et al., 2001).

An advantage of our protease characterization system, either with substrate-phage selection or with fluorescent recombinant substrates, is that cleavage of the substrate occurs in a protein context. Thus, the search for potential biological substrates using selected peptides appears to be more relevant than with chemical substrates. However, whereas substrate phage-display selection of peptides identifies high-affinity substrates, natural target sequences may differ from selected peptide sequences due to potential additional exosites or other protein-protein interactions. Therefore, proteins identified by database searches that contain sequences homologous to selected pentapeptides should be confirmed to be real protease targets *in vitro*. Several sites, particularly in extracellular matrix (ECM) proteins, seem plausible enough to suggest further experiments. Thus, we tested hydrolysis of ECM proteins under hK14 proteolytic activity. The most interesting ECM protein tested was laminin α-5 chain, which contains a LSRDN sequence identical to that of clone E7. Laminin α-5 chain is a part of laminin 10/11, which is a strong adhesive complex for normal and malignant human epithelial cells. We showed that hK14 can cleave efficiently the laminin α-5 chain at a low enzyme concentration. Under the same conditions, collagen IV was also efficiently degraded. This multiple fragmentation indicates that there might be more than one potential hK14 cleavage site in collagen IV. Taken together, these proteolytic activities could contribute to the invasive behavior of breast and ovarian cancer cells, which are known to secrete hK14 (Borgono et al., 2003). Other human kallikreins have already been suspected to favor migration of cancer cells, such as hK2 (Deperthes et al., 1996; Frenette et al., 1997b) and PSA (Webber et al., 1995).

Some other potential biological substrates have been identified from the peptides selected. One of them occurs in a little-known protein named matrilin-4, which is a non-collagenous extracellular matrix protein. This protein is expressed during embryogenesis and at the developing joint surface (Klatt et al., 2002) but its role and the consequence of its proteolytic degradation remains to be determined.

Endothelin-2 (ET-2) precursor could also be a target for hK14 proteolysis, with a substrate sequence in its C-terminal part. ET-2 is produced endogenously from preproendothelin to generate big endothelins, which are cleaved by endothelin-converting enzyme (ECE) to yield the active peptide (Goraca, 2002). Endothelin has been shown to play important physiological roles by interacting with its G-protein-coupled receptors. The overall action of endothelin is to increase blood pressure and vascular tone. The potential cleavage site of ET-2 by hK14 is not within the sequence of the active protein. However, the proteolysis of the C-terminal part of ET-2 could either prevent activation of the precursor by ECE or could liberate a new form of active ET-2.

The multidomain serine proteinase inhibitor Kazal-type 5 precursor, also named LEKTI, was also identified as a potential target for hK14. Domain 15, which shares significant sequence homology with an inhibitor of tryptase (Magert et al., 2002), LDTI, could be cleaved by hK14. The role of LEKTI still remains to be clarified, but its relation with the severe congenital disorder Netherton syndrome, due to the presence of mutations, demonstrates its biological importance.

Finally, two additional identical matches occur either in proteins that are expected to be intracellular (proprotein convertase subtilisin) or in a signal peptide (airway trypsin-like precursor) and, thus, unavailable for cleavage by hK14.

In summary, we have used phage-display technology to identify the first hK14 substrate sequences. We have demonstrated that hK14 displays extended substrate specificity, with trypsin- and chymotrypsin-like activities. Moreover, we showed that hK14 can degrade some components of extracellular matrix proteins with high efficacy. However, further studies will be necessary to verify if any of the proteins identified represent the physiological substrates for hK14.

Materials and methods

Materials

The following materials were obtained from commercial sources: elastase, trypsin, chymotrypsin, and plasma kallikrein (Calbiochem, Lucerne, Switzerland), human laminin 10/11 (Chemicon, Lucerne, Switzerland), human collagen IV (Life Technologies, Buchs, Switzerland), T4 DNA ligase (Invitrogen, Carlsbad, USA), T4 polynucleotide kinase (Qbiogene, Basel, Switzerland), Ni^{2+}-nitrilotriacetic acid agarose beads (Qiagen, Basel, Switzerland), restriction enzymes (Roche, Mannheim, Germany; Amersham

Pharmacia, Piscataway, USA; Promega, Wallisellen, Switzerland), and anti-His antibody (Sigma, Buchs, Switzerland). Oligonucleotide synthesis was carried out by Invitrogen (Basel, Switzerland) and DNA sequencing by Synergene Biotech GmbH (Schlieren, Switzerland). Human kallikrein 2 and prostate-specific antigen were purified from human seminal plasma as previously described (Frenette et al., 1997a, 1998).

Cloning of *KLK14* into *Pichia pastoris* expression vector pPICZαA

First-strand cDNA synthesis was performed by reverse transcriptase using the Superscript™ preamplification system (Gibco BRL, Gaithersburg, MD, USA) with 2 μg of total human cerebellum RNA (Clontech, Palo Alto, CA, USA) as a template. The final reaction volume was 20 μl. To confirm the efficiency of RT-PCR, 1 μl of cDNA was subsequently amplified by PCR with primers specific for *actin*, a housekeeping gene (ActinS: 5′-ACA ATG AGC TGC GTG TGG CT; ActinAS: 5′-TCT CCT TAA TGT CAC GCA CGA). Actin PCR products with an expected length of 372 bp were visualized on a 2% agarose gel stained with ethidium bromide.

PCR amplification of *KLK14* cDNA encoding the 227 amino acids of the mature hK14 protein (corresponding to amino acids 25–251 of GenBank accession no. AAK48524) was carried out in a 50-μl reaction mixture containing 1 μl of cerebellum cDNA as a template, 100 ng of primers (FPL6: 5′-AGG ATG AGG AAT TCA TAA TTG GTG GCC AT; RPL6: 5′-CCC ACC GTC TAG ACC ATC ATT TGT CCC GC), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 200 μM deoxynucleoside triphosphates (dNTPs) and 0.75 μl (2.6 U) of Expand Long Template PCR polymerase mix (Roche Diagnostics), using an Eppendorf master cycler. The PCR conditions were: 94°C for 2 min, followed by 94°C for 10 s, 52°C for 30 s, and 68°C for 1 min for 40 cycles, and a final extension at 68°C for 7 min. Following PCR, amplified *KLK14* was visualized with ethidium bromide on 2% agarose gels, extracted, digested with *Eco*RI/*Xba*I and ligated into expression vector pPICZαA of the Easyselect™ *Pichia pastoris* expression system (Invitrogen) at the corresponding restriction enzyme sites using standard techniques (Sambrook et al., 1989). The *KLK14* sequence within the construct was confirmed with an automated DNA sequencer using vector-specific primers in both directions.

Protein production

*Pme*I-linearized pPICZαA-*KLK14*, as well as empty pPICZαA (negative control), were transformed into the chemically competent *P. pastoris* yeast strain X-33, after which they integrated into the yeast genome by homologous recombination. Transformed X-33 cells were then plated on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar) plates containing Zeocin™, a selective reagent. A stable yeast transformant was selected according to the manufacturer's recommendations, inoculated in buffered minimal glycerol-complex (BMGY) medium [1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6.0), 1.34% yeast nitrogen base, 40 mg/l biotin, and 1% glycerol] overnight at 30°C on a plate agitator at 250 rpm, diluted to OD_{600}=1.0 in BMMY (same as BMGY except that 1% glycerol is replaced with 0.5% methanol) and incubated under the same conditions as above for 6 days with a daily supplement of 1% methanol. The supernatant was collected by centrifugation at 4000 *g* for 20 min.

Protein purification

Recombinant hK14 was purified from yeast culture supernatant by cation exchange using a 5-ml HiTrap™ carboxymethyl (CM) Sepharose Fast Flow column on the ÄKTA FPLC chromatography system (Amersham Biosciences). First, the supernatant was filtered with a 0.22-μm disposable filter and concentrated 50-fold by ultrafiltration with an Amicon™ YM10 membrane (Millipore Corporation, Bedford, MA, USA). The filtered, concentrated supernatant was then introduced into the injector of the ÄKTA FPLC system and loaded onto the CM sepharose column, previously equilibrated with 5 ml of 10 mM 2-(*N*-morpholino)ethane sulfonic acid (MES) buffer (pH 5.3) at a flow rate of 0.8 ml/min. The column was washed with the aforementioned equilibration buffer and the adsorbed hK14 was eluted with a 150-ml continuous linear KCl gradient from 0 to 1 M in 10 mM MES (pH 5.3) at a flow rate of 3 ml/min. Elution fractions of 5 ml were collected and analyzed. Fractions containing hK14 were pooled and further concentrated 10 times using a Biomax-10 Ultrafree®-15 centrifugal filter device (Millipore). The protein concentration of the purified hK14 was determined by the bicinchoninic acid method (Smith et al., 1985), which uses bovine serum albumin as calibrator (Pierce Chemical Co, Rockford, IL, USA). The purity of the recombinant hK14 protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) followed by Coomassie Brilliant Blue staining and/or Western blot analysis using a previously produced polyclonal rabbit antibody raised against hK14 (Borgono et al., 2003) and its identity was confirmed by tandem mass spectrometry, as described in detail for recombinant hK10 (Luo et al., 2001).

Phage-displayed pentapeptide library screening

A phage-display substrate library displaying pentapeptides (Cloutier et al., 2002) was subjected to six rounds of screening with hK14. Briefly, substrate phages (10^{11}) were incubated with 60 μl of Ni^{2+}-nitrilotriacetic acid resin in phosphate-buffered saline (PBS) 1× containing bovine serum albumin (BSA) at 1 mg/ml, washed four times (PBS 1×, 1 mg/ml BSA, 5 mM imidazole, 0.1% Tween 20) to remove unbound phages and then exposed to 65 nM (final concentration) of hK14 for 45 min at 37°C in 50 mM Tris, 100 mM NaCl, 0.05% Triton, pH 7.5. The released phages were subsequently amplified using XL1-Blue *Escherichia coli* and then used after purification for subsequent rounds of selection. A total of 32 individual clones from the last round of selection were sequenced for determination of the corresponding amino acid sequences.

Expression of CFP-YFP fluorescent substrate

Recombinant fluorescent substrates, using cyan fluorescent protein as donor and yellow fluorescent protein as acceptor, were constructed as recently described (Felber et al., 2004). CFP-**XXXXX**-YFP-6×His recombinant proteins were constructed with varying pentapeptides (in bold) between CFP and YFP proteins using synthetic genes possessing the appropriate restriction sites (*Bss*HII; *Sal*I). The constructs contain the following amino acid sequences between CFP and YFP proteins: Gly-Ala-Leu-Gly-Gly-**XXXXX**-Gly-Ser-Thr. To produce recombinant proteins, TG1 cells were transformed with the corresponding constructs and purified by affinity chromatography using Ni^{2+}-NTA agarose beads. The purity and quantity of the purified CFP-YFP recombinant substrates were evaluated by SDS gel electrophoresis according to Laemmli (1970) followed by Coomassie Brilliant Blue staining and Western blot analysis using a specific anti-His primary antibody (1/3000 dilution), a mouse anti-Fab secondary antibody (1/50 000 dilution) and the ECL system (Amersham) for detection. All clones were sequenced prior to evaluation.

Direct determination of the k_{cat}/K_m value and specificity studies using CFP-YFP fluorescent substrates

The substrate specificity of CFP-substrate-YFP proteins was tested towards different proteases and k_{cat}/K_m values were calculated as previously described (Felber et al., 2004). Briefly, fluorescence of CFP-X_5-YFP proteins was measured in black 96-well plates using a microplate fluorescence reader (Bio-Tek Instruments Inc, Winooski, VT, USA) with excitation at 440 nm (±15) and emission at 485 (±10) and 528 nm (±10). Each recombinant substrate, at a concentration of 150 nM, was incubated with hK14, chymotrypsin, trypsin, PSA, hK2, plasma kallikrein or elastase at a final concentration of 8 nM, 0.1 nM, 0.3 nM, 2 μM, 10 nM, 10 nM and 0.5 nM, respectively. The reaction was carried out for 60 min at 37°C in reaction buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 0.05% Triton X-100). The enzyme concentration for initial-rate determinations was chosen at a level intended to hydrolyze specifically the substrate linker and not a GGGGG substrate, which was used as negative control. The appearance of fluorescence, corresponding to product formation, was measured spectrometrically with excitation at 440 nm (±15) and emission at 485 nm (±10). The slope was converted into units of nmol of product generated per second, based on a calibration curve obtained from the complete hydrolysis of each peptide, evaluated on SDS-PAGE. The kinetic parameter k_{cat}/K_m was determined under pseudo-first-order conditions using a substrate concentration far below the estimated K_m (Felber et al., 2004).

The cleavage products were separated by SDS-PAGE, transferred to an Immobilon polyvinylidene difluoride membrane (Bio-Rad, Reinach, Switzerland), and subjected to automated Edman degradation with an Applied Biosystems (model ABI493A) sequenator to determine the cleavage site.

Hydrolysis of laminin and collagen IV

Extracellular matrix proteins, 5 μg of human laminin 10/11, 7.7 μg of type IV collagen, or 1 μg of BSA, were incubated for 3 or 12 h at 37°C with or without 200 nM of hK14 in PBS at pH 7.4 for laminin and 300 mM acetic acid, 300 mM Tris, 100 mM NaCl at pH 7.3 for collagen. Samples were subsequently analyzed by SDS-PAGE under reducing conditions on a 4–15% Tris-HCl gradient gel (Bio-Rad), and gels were stained with Coomassie Brilliant Blue R250 followed by silver nitrate.

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