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Substrate specificity determination of mouse implantation serine proteinase and human kallikrein-related peptidase 6 by phage display*

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Abstract

We constructed a random library of hexapeptides displayed on the surface of bacteriophage T7 to determine the substrate specificity of proteinases. The phage-displayed library was subjected to repeated rounds of biopanning with native implantation serine proteinase and recombinant human kallikrein-related peptidase 6 (KLK6) followed by selection and identification of putative substrates. For both enzymes, the results obtained demonstrate a preference for arginine and lysine at multiple positions in the recognition cleavage motif, confirming their previously reported trypsin-like substrate specificity. In the case of KLK6, there is also a pronounced presence of tryptophan within the cleaved peptide sequences, indicating its potential dual substrate specificity, acting as both a trypsin and chymotrypsin-like enzyme.

Keywords: enzyme specificity; human kallikrein-related peptidase; implantation serine proteinase; phage display; T7 bacteriophage.

Introduction

Proteinases represent one of the most versatile groups of enzymes, represented by a large number of families in different species (Puente et al., 2003). They have been implicated in many different pathways involved in various physiological, as well pathological settings. Among the different classes of proteinases, serine proteinases are the best-known molecular scissors devised by nature. However, determining the exact cleavage site(s) in native proteins has always been a challenge. To deal with this challenge, different high throughput screening methods have been used to determine the substrate specificity, including synthesizing libraries of chromogenic substrates (*p*-nitroanilide-conjugated peptides) and fluorogenic substrates (7-amino-4-methycoumarin-conjugated peptides), either in solution or on membranes/chips. The display of small peptides on the surface of bacteriophage, also known as phage display, has been developed as a more efficient and unbiased way of determining the specificity of a proteinase. Thus far, this technology has been exploited for displaying libraries of peptides/proteins for many diverse applications, including but not limited to affinity selection, generating specific antibodies and elucidating the substrate specificity of proteinases (Smith, 1985; McCafferty et al., 1990; Matthews and Wells, 1993).

Previously, the T7 bacteriophage display system has been employed successfully for determining the substrate specificities of rat mast cell proteinases 4 and 5, which exhibit chymase-like and elastase-like enzyme activity, respectively (Karlson et al., 2002, 2003). In the work described here, we employed this approach to display random hexameric peptides to determine the substrate specificity of two native, uterine localized serine proteinases, viz. murine implantation serine proteinase (ISP) (Sharma et al., 2006) and recombinant human kallikrein-related peptidase 6 (KLK6) (Oikonomopoulou et al., 2008). It is our working hypothesis that both of these enzymes play an important role in the processes of embryo implantation in the uterus and placental maintenance, but with different target substrate specificity. Both enzymes are secreted serine proteinases of the S1 family, according to the MEROPS database [an information resource for peptidases (also termed proteases, proteinases and proteolytic enzymes) and their inhibitors (http://merops.sanger.ac.uk/; Rawlings et al., 2006)] classification. The target specificities of these enzymes for naturally occurring substrates have yet to be evaluated in depth.

The ISP genes, ISP1 and ISP2, are differentially expressed in the uterine endometrium and blastocyst during peri-implantation (O'Sullivan et al., 2001, 2002; Sharma et al., 2006). They are known to play a critical role in the processes of embryo hatching and implantation (O'Sullivan et al., 2001, 2002; Huang et al., 2004; Sharma et al., 2006). Recently, we have purified ISP from murine uterus as a hetero-dimeric ISP1-ISP2 complex, which is the native, functional form of this enzyme (Sharma et al., 2006). Preliminary studies using chromogenic *p*-nitroanilide conjugated synthetic substrates have suggested that the ISP enzyme complex has 'trypsin-like' specificity (Sharma et al., 2006).

KLK6 is a member of the large family of human kallikrein-related peptidases expressed in the uterus and in

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various other normal and cancerous tissues, including the ovary, gastrointestinal tract and central nervous system (Borgoño and Diamandis, 2004; Borgoño et al., 2004; Yousef et al., 2004; Shaw and Diamandis, 2007). KLK6 has been implicated as a prognostic or diagnostic biomarker for many types of cancer, including that of uterus, ovary and breast (Hoffman et al., 2002; Diamandis et al., 2003; Borgoño and Diamandis, 2004; Santin et al., 2005). KLK6 has been shown to cleave efficiently *in vitro* many carcinogenesis-related substrates, including a variety of extracellular matrix proteins (Magklara et al., 2003; Borgoño and Diamandis, 2004; Ghosh et al., 2004). Recently, KLK6 has been found to play an important role in proteinase-activated receptor mediated signaling, increasing the number of possible functional roles for this proteinase (Oikonomopoulou et al., 2006a,b).

The ‘trypsin-like’ substrate specificity of KLK6 is well accepted, based on structural and phylogenetic studies (Bernett et al., 2002) and on the preliminary screening of synthetic fluorogenic peptides (Magklara et al., 2003). Furthermore, Angelo et al. (2006) reported the substrate specificity of KLK6 along with the effect of salts and glycosaminoglycans by using a fluorescence resonance energy transfer approach, employing variants of a parent peptide based on the subsite requirements for KLK1 substrates. The most convincing result in the study was the selectivity of KLK6 for arginine and lysine at the P1 position, with a preference for arginine more highly pronounced in comparison to lysine. The results obtained for the specificity of KLK6 for positions other than P1 (viz. P2, P3, P1′, P2′, P3′, etc.) were not definitive. That said, Debela et al. (2006), using a positional scanning combinatorial library of tetrapeptides as substrates, proposed a strong preference of KLK6 for arginine at the P1, as well as at the P2 position.

Phage display has also been employed for determining the specificity of human KLK14 by using a library of pentapeptides on the surface of M13 bacteriophage (Felber et al., 2005). The results obtained, which are complementary to other means of determining proteinase specificity, profiled the specificity of KLK14 as a mixed one with both trypsin-like as well as chymotrypsin-like activities. Considering the potential of the phage-display approach and the importance of both ISP and KLK6 in different physiological and pathological processes, we sought to explore and compare their substrate specificity in more detail using a random phage-displayed library of large diversity.

Results and discussion

Substrate specificity determination of implantation serine proteinase complex

By virtue of having the $(His)_6$ tag, the phage particles were bound to the Ni-NTA-agarose beads followed by biopanning with a purified, active ISP enzyme complex preparation. The cleaved phage particles thus obtained were amplified and titrated by plating on a lawn of bacteria grown (BLT5403) in agarose medium. Figure 1A shows the titration results of phage particles obtained after every cycle of biopanning (non-amplified) in comparison to the control (phage particles were eluted from Ni-NTA beads using 0.5 M imidazole hydrochloride). The procedure was repeated five times and a total of 21 phage plaques were selected for sequencing of the random hexameric region following PCR amplification. The non-aligned unbiased amino acid sequence, deduced from the DNA sequence of the random hexameric insert region, obtained from each plaque, is listed in Table 1.

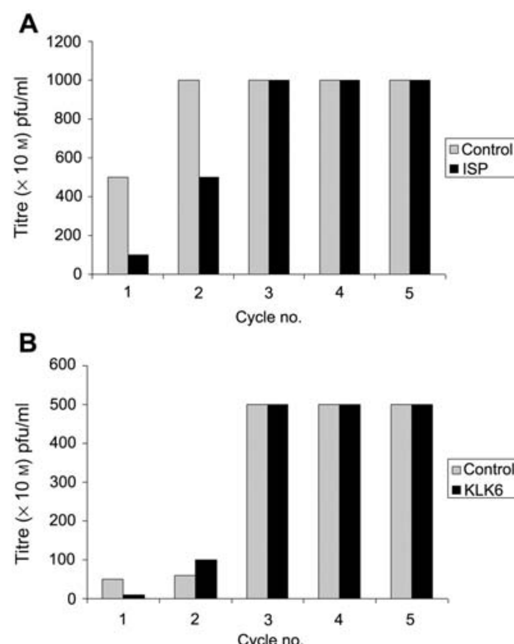
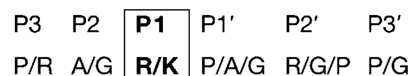


Figure 1 Titration results of the non-amplified phage plaque preparations obtained after consecutive rounds of biopanning.
Titration and biopanning were carried out as outlined in the materials and methods section. (A) Biopanning with ISP enzyme complex. (B) Biopanning with recombinant human kallikrein 6 (KLK6). Control data: gray bars; ISP/KLK6-generated data: black bars.

The data show that out of 21 hexapeptides, 15 had an arginine/lysine residue in their sequences (Table 1, bold italic letters). We previously reported the ‘trypsin-like’ specificity of the ISP enzyme complex, determined by using chromogenic *p*-nitroanilide substrates (Sharma et al., 2006). The biopanning data presented here validate our earlier finding by demonstrating the presence of arginine/lysine residues in 72% of the phage plaques. Therefore, these plaque sequences were aligned assuming an arginine/lysine residue at the P1 position. The frequency of occurrence of an amino acid at a given position (i.e., P1, P1′, P2…) in comparison to its random probability is shown in Figure 2. Based on the above analysis, the following recognition cleavage sequence motif was identified for the native ISP enzyme complex:



These results demonstrate a preference for non-polar amino acids at positions in the vicinity of cleavage site, with the neighboring P1′ and P2 positions occupied by proline/alanine/glycine and alanine/glycine, respectively. The other sites (P2′, P3′ and P3) also indicate a preference for non-polar amino acids, along with arginine at P3

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Table 1 Plaque sequences of random hexamer region obtained after biopanning with ISP enzyme complex.

Plaque no.	AA1	AA2	AA3	AA4	AA5	AA6
1	–	V	G	P	***R***	***R***
2	F	L	L	C	E	Q
3	***R***	V	P	M	A	***R***
4	***R***	W	P	E	L	E
5	P	***R***	V	***K***	G	A
6	A	D	G	***R***	A	V
7	G	M	V	G	Q	G
8	H	G	***R***	***K***	***R***	***R***
9	G	S	W	S	S	M
10	***R***	A	***R***	A	T	M
11	L	W	***R***	G	P	***K***
12	A	V	V	L	L	S
13	S	***R***	G	***R***	L	G
14	G	Y	G	V	D	A
15	V	***R***	S	L	I	F
16	***R***	D	***R***	L	P	P
17	P	G	S	***R***	E	***R***
18	Q	V	D	Q	G	S
19	G	***R***	-	V	N	G
20	P	E	***R***	C	W	M
21	***R***	C	I	T	A	***R***

The presence of arginine and lysine residues in the random hexapeptide region of the plaque sequence is indicated by bold italics (***R***, ***K***).

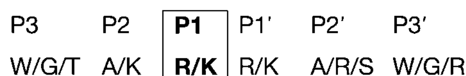
and P2′. By comparing these results with the MEROPS peptidase database, we found that there are multiple serine proteinases preferring either Ala at P2 or Pro at P1′, but not many proteinases are known to target substrates with Ala at the P2 position and Pro at P1′. Therefore, according to our analysis, ISP appears to have unexpectedly unique substrate specificity. This specificity will need to be validated by further work with synthetic peptide substrates, based on our predicted consensus cleavage sequence motif described above.

To identify putative substrates of ISP, this consensus amino acid sequence was compared in different permutations and combinations with the National Center for Biotechnology Information (NCBI) non-redundant database. A list of probable ‘hits’ obtained with a high degree of confidence (80–100% identity) is shown in Table 2. The hits obtained reveal potential target substrates within the extracellular matrix/membrane protein families, including some receptors and different procollagen types. The list shown here is indicative but not complete. In further studies, we plan to repeat the phage-display experiments using recombinant active ISPs produced in a methylotrophic strain of *Pichia pastoris* and to test the susceptibility of the ‘hit’ proteins listed in Table 2 to cleavage by ISP.

Substrate specificity determination of human kallikrein-related peptidase 6

Recombinant, active human KLK6 was produced from cell culture supernatants using ion exchange chromatography as described elsewhere (Oikonomopoulou et al., 2008; modified from Magklara et al., 2003) and was subjected to phage-display analysis using optimum hydrolysis conditions (25 mM Tris-HCl buffer, pH 8.0, 37°C, overnight). Figure 1B shows the titration results of phage particles obtained after every cycle of biopanning (non-amplified) in comparison to the control (phage particles were eluted from Ni-NTA beads using 0.5 M imidazole hydrochloride). A total of 32 phage plaques were sequenced for the random hexameric region after PCR amplification. The non-aligned unbiased amino acid sequences of the random hexameric region obtained from each plaque are listed in Table 3. As expected, the results demonstrate that KLK6 has a high preference for arginine/lysine, in accordance with the results obtained previously by Magklara et al. (2003) and Angelo et al. (2006). This preference is illustrated by the high frequency of occurrence of arginines and lysines in Table 3 (bold font). More specifically, not only did most of the plaque sequences (29 out of 32) have at least one arginine/lysine residue, approximately 60% of the plaque sequences (20 out of 32) had more than one arginine/lysine residue in the random hexapeptide displayed. The trypsin-like substrate specificity of KLK6 can be compared to the trypsin-like activity of the ISP enzyme complex (Sharma et al., 2006).

To evaluate the ability of KLK6 to cleave a synthetic peptide, we used the non-aligned phage-display data (Table 3) to prepare the following peptide substrate: (GGRRRKVGG) in which the target sequence is underlined and in which two glycines were added at the N- and C-terminus to facilitate efficient HPLC separation and matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy determination of the masses of the cleavage products. This peptide proved to be a satisfactory substrate for KLK6, yielding four cleavage products (Figure 3), indicating a cleavage of the peptide at each available P1 arginine residue, but not at a possible P1 lysine residue (potential cleavage products, GGRRRK and VGG, not found). These preliminary data suggest a preference for a basic residue at both the P1 and P1′ positions, akin to a furin-like enzyme recognition motif. Based on our peptide cleavage data, the plaque sequences were therefore aligned assuming an arginine/lysine residue at the P1 position, according to the data shown in Table 3. The percent frequency of an amino acid at a given position (i.e., P1, P1′, P2…) in comparison to its random probability is shown in Figure 4. Upon analyzing these data, the following recognition sequence was identified as a potential cleavage motif for recombinant human KLK6:



Our data suggest that there is a higher preference for alanine followed by lysine at the P2 position. Neutral amino acids are preferred at the P3 position, with the presence of tryptophan being most highly pronounced. Similarly, there is a high preference for alanine (followed by arginine and serine) at the P2′ position and tryptophan (followed by glycine and arginine) at the P3′ position. Furthermore, as with the P1 position, arginine/lysine are also preferred in the P1′ position. Based on these results the preferred P1-P1′ scissile bond appears to be a dibasic arginine-arginine/arginine-lysine/lysine-arginine doublet. In keeping with our analysis of the cleavage of the synthetic peptide, GGRRRKVGG, and with the work of

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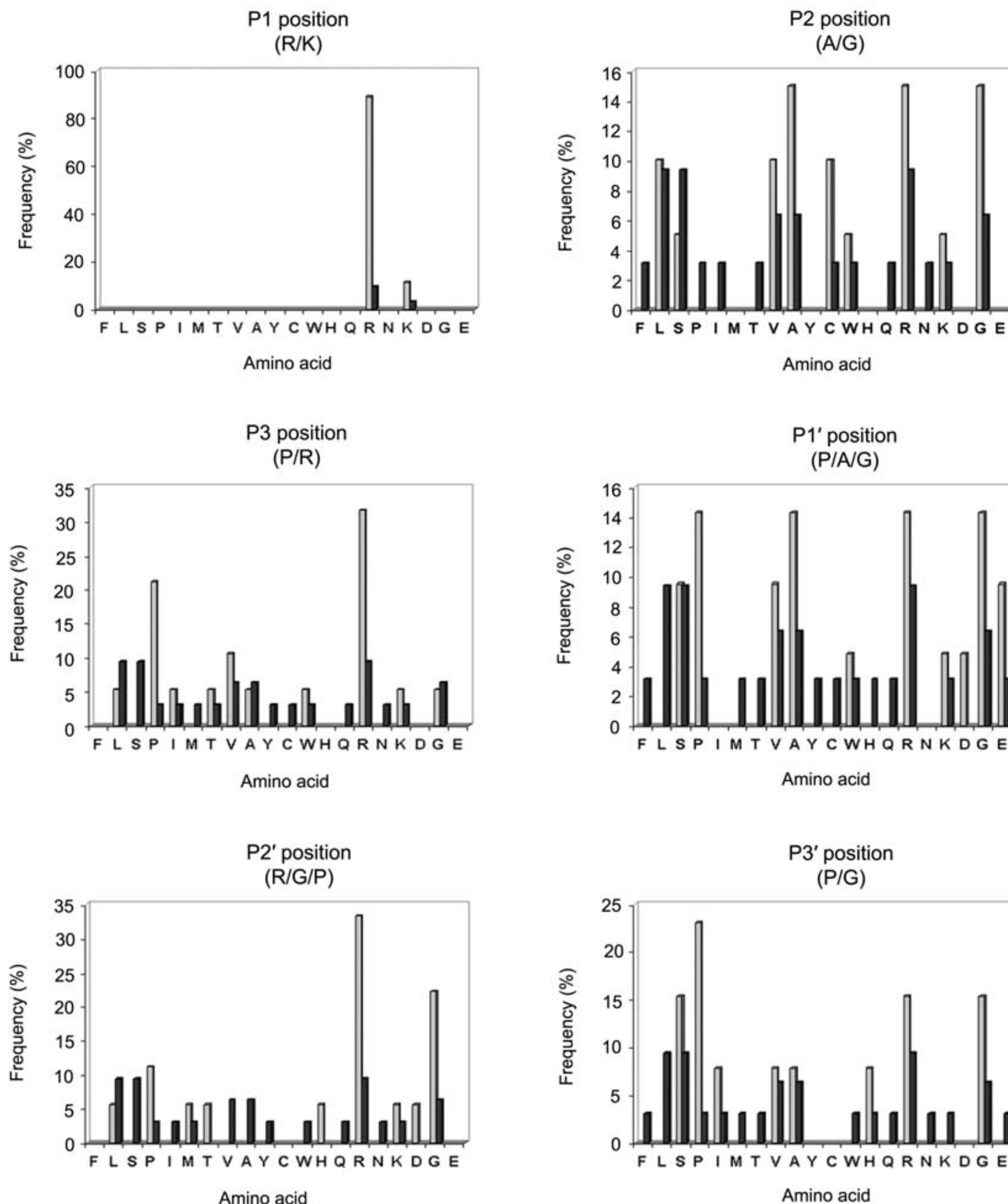

Figure 2 Biopanning results of T7 bacteriophage-displayed library of random peptides (6-mer) with purified ISP enzyme complex. The frequency of occurrence of amino acid residues at a given position (P1, P2, P3, P1′, P2′ and P3′) after alignments assuming arginine/lysine at the P1 position (% frequency, y-axis) is shown. The observed frequencies (gray bars) are shown in comparison to the frequencies expected in the absence of any bias (black bars).

Debela et al. (2006), the predicted preference for doublets of basic R/K residues at the cleavage site of KLK6 substrates resembles the pro-protein processing function of furin and other pro-protein convertases. This type of substrate specificity would make KLK6 a possible candidate for activating membrane-tethered matrix metalloproteinases (MT-MMPs) (Pei and Weiss, 1995; Sternlicht and Werb, 2001). That said, a high abundance of tryptophan at the P3 and P3′ positions also suggests the possibility that KLK6 exhibits some 'chymotrypsin-like' activity, in keeping with what has been reported for another human kallikrein-related peptidase family member, KLK14 (Felber et al., 2005). In contrast, a phage-display approach showed that KLK2 exhibits a trypsin-like activity with a preference for arginine at the P1 position, but without appreciable chymotrypsin-like activity (Cloutier et al., 2002). These substrate preferences suggested for KLK6 by our phage-display approach merit validation using synthetic peptides, based on the predicted recognition hexamer motif described above.

A screening of the MEROPS peptidase database identified 44 proteinases, including mouse kallikrein 1 and human proprotein convertase 2, which have a substrate specificity similar to that predicted by our phage-display approach for the P1-P1′ positions. Upon extending the search to the P2-P1-P1′-P2′ positions predicted by our recognition motif analysis, the MEROPS database yielded only one proteinase, namely Omptin (an *Escherichia coli* outer membrane serine endopeptidase) with a similar specificity to KLK6.

To identify putative substrates of KLK6 apart from the MT-MMPs, the consensus target amino acid sequence yielded by the phage-display approach was compared to

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Table 2 Potential putative substrates of the ISP enzyme complex.

Putative substrate	Accession no.	Cleavage motif
1. Plexin C1	gb|EDL21590.1|	96 PARPRP 101
2. Leucine rich repeat and fibronectin type III domain containing 1	dbj|BAE53711.1|	690 PARPRP 695
3. SALM2	gb|AAZ20639.1|	690 PARPRP 695
4. Pleckstrin homology domain-containing, family A member 4	ref|NP_683729.1|	87 PARPRP 92
5. Receptor for viral-encoded semaphorin protein	gb|AAF01334.1|AF190578_1	96 PARPRP 101
6. Cholecystokinin B receptor	gb|AAI03531.1|	419 RARPRP 424
7. Procollagen, type V, α1	gb|EDL08374.1|	1104 PGRPGP 1109 903 PGKPGP 908 1431 PGKPGP 1436
8. Procollagen, type XI, α1	gb|EDL12408.1|	1047 PGRPGP 1052
9. Procollagen, type IV, α3	gb|EDL02136.1|	950 PRPGP 955 1057 PGKPGP 1062
10. Procollagen, type IV, α4	gb|EDL02135.1|	1230 PGRPGP 1235

Table 3 Plaque sequences of random hexamer region obtained after biopanning with human KLK6.

Plaque no.	AA1	AA2	AA3	AA4	AA5	AA6
1	L	***R***	A	A	F	Q
2	V	L	W	T	L	***R***
3	***K***	D	L	L	***K***	C
4	G	G	F	T	H	V
5	Y	***R***	S	V	E	W
6	***R***	T	A	T	G	***R***
7	***K***	***R***	S	T	V	***R***
8	L	***R***	***R***	G	G	V
9	V	A	A	***R***	S	A
10	V	M	V	***R***	S	V
11	M	T	F	***R***	S	A
12	A	***R***	S	I	***R***	V
13	S	***R***	A	***R***	S	A
14	G	E	S	T	H	G
15	V	E	V	A	***K***	D
16	W	F	D	N	T	M
17	S	***R***	A	***R***	S	A
18	Q	***R***	G	V	L	***R***
19	A	***R***	***R***	W	***R***	***R***
20	***R***	A	***R***	L	***R***	Q
21	S	G	W	***R***	V	G
22	***R***	F	***R***	Q	***K***	F
23	W	***K***	***R***	Q	***R***	W
24	N	***R***	***R***	S	W	***K***
25	E	***R***	S	***R***	***R***	S
26	G	G	W	***R***	***K***	A
27	Y	N	***R***	M	A	G
28	Y	S	S	***K***	***R***	A
29	***R***	***R***	***R***	G	N	G
30	L	G	V	***R***	A	***R***
31	Q	V	T	***R***	***K***	V
32	A	***R***	G	S	***R***	G

The presence of arginine and lysine residues in the random hexapeptide region of the plaque sequence is denoted by bold italics (***R***, ***K***).

the NCBI non-redundant database in different permutations and combinations. A list of probable 'hits' obtained with a high degree of confidence (80–100% identity) is shown in Table 4. These potential substrates include many membrane-bound and extracellular proteins, such as the prostaglandin E2 and γ-aminobutyric acid (γ-GABA) receptors, fibrillin 1 and protocadherin. The list of targets is of course hypothetical and must be confirmed by more direct studies which are in progress in our laboratory.

In summary, with the data presented here we demonstrated the use of phage display of small peptides as a fruitful approach for a preliminary determination of the differential substrate specificity of the ISP and KLK6 enzymes. This initial study points to interesting differences in the substrate specificities of these two enzymes, both of which exhibit trypsin-like activity, with a putative preference of R/K at the P1 site, but each of which has a distinct predicted cleavage motif comprising different

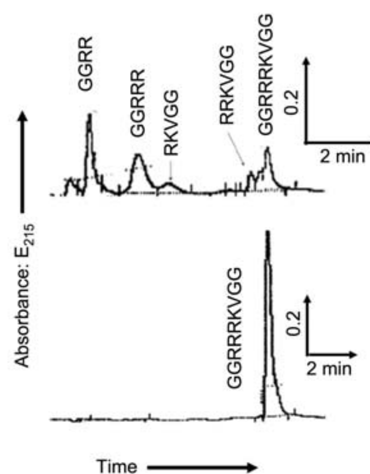

Figure 3 *In vitro* cleavage of a synthetic peptide by KLK6 and HPLC separation of the peptide hydrolysis products for MALDI-TOF mass spectrometric analysis.
Lower panel: HPLC chromatogram of intact peptide GGRRRKVGG; upper panel: HPLC chromatogram showing cleavage products obtained upon hydrolysis of GGRRRKVGG after incubation with KLK6, as determined by MALDI-TOF mass spectrometry. The scales for time (minutes) and absorbance (E_{215}: arbitrary absorbance units) are shown by the inserts (arrows) to the right of each chromatogram. The peptide hydrolysis sequences deduced by MALDI-TOF analysis are shown above each peak in the upper chromatogram. The peak for the synthetic peptide substrate shown in the lower chromatogram corresponds to a minor peak in the chromatogram of the hydrolysis products shown in the upper chromatogram.

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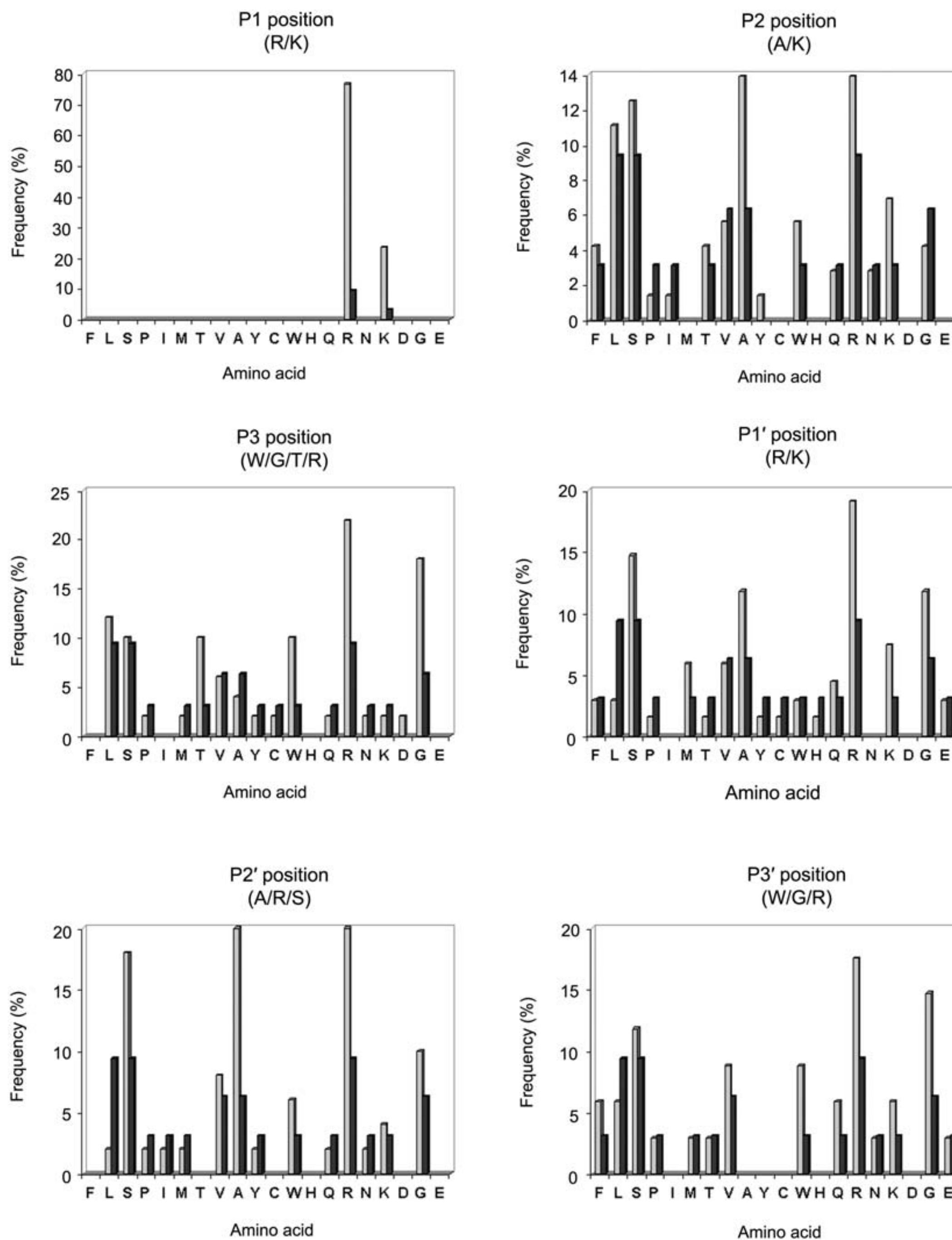

Figure 4 Biopanning results of T7 bacteriophage-displayed library of random peptides (6-mer) with purified recombinant KLK6.
The frequency of occurrence of amino acid residues at a given position (P1, P2, P3, P1′, P2′ and P3′) after alignments assuming arginine/lysine at the P1 position (% frequency, y-axis) is shown. The observed frequencies (gray bars) are shown in comparison to the frequencies expected in the absence of any bias (black bars).

preferences for amino acids at the P2, P3 and P1′, P2′ and P3′ sites. Further, our data indicate a similarity in properties between KLK6 and KLK14 in that both of these kallikrein-related peptidase family members (unlike other members of the family studied in depth to date) display both tryptic and chymotryptic activities. Continuing work is required to explore in more detail the catalytic properties of the ISP and KLK6 enzymes (e.g., k_{cat}/K_m values) for hydrolysis of the peptide sequences we identified as preferred target motifs. Continuing work will also be necessary to validate the complete list of possible substrate proteins extracted from the bioinformatic analysis and to evaluate *in vitro* cleavage of the synthetic peptides and target substrate proteins identified by us, by employing HPLC and mass spectrometry techniques.

Materials and methods

Construction of T7 phage-displayed peptide library and its validation using thrombin

A library of random hexapeptides (6-mers) was generated as described by Karlson et al. (2002) with minor modifications. The sequence of synthetic degenerate oligonucleotides inserted in the coding region of T7 phage capsid protein (employing T7

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Table 4 Potential putative substrates of human KLK6.

Putative substrate	Accession no.	Cleavage motif
1. Prostaglandin E2 receptor	gb|AAA61681.1|	246 GARRRG 251
2. Potassium channel tetramerization domain containing 8	ref|NP_938167.1|	82 GARRRG 87
3. Saposin-like protein	gb|AAK38148.1|	19 WARRS 23
4. Protocadherin 15	emb|CAM15117.1|	1502 WARKR 1506
5. γ-Aminobutyric acid (GABA) receptor, rho 2 precursor	ref|NP_002034.2|	49 KRKRW 53
6. Fibrillin 1 precursor	ref|NP_000129.2|	42 AKRRG 46
7. SCRL protein	gb|AAQ05826.1|AF479645_1	141 AKRRG 145
8. G protein-coupled receptor	gb|AAL26488.1|AF411117_1	290 WKKKR 294
9. Erythrocyte membrane protein band 4.1 (elliptocytosis 1, RH-linked)	emb|CAI21969.1|	405 WKKKR 409

Select 1-1 vector arms, T7 Select system, Novagen Canada: EMD Biosciences, Inc., an affiliate of Merck KGaA, Darmstadt, Germany), encoding a random hexamer followed by $(His)_6$ tag is shown here: 5′-AAT TCT CTC ACT CCA GGC GGC-$(NNK)_6$-GGT GGT CAT CAC CAT CAC CAT CAC TAA-3′ (N represents any nucleotide and K represents T or G). The total library size was estimated to be 1.5×10^8 pfu/ml based on number of transformants. Therefore, the library represented almost all the possible combinations of random hexamers (6.4×10^7) as published by Deperthes (2002). The diversity of the library and unbiased incorporation of nucleotides and hence amino acids into the hexameric peptide region was confirmed by sequencing randomly picked phage plaques and determining the ratio of observed versus expected frequency as published by Cwirla et al. (1990) (Tables 5 and 6). Subsequently, the library was amplified to a titer of 1.0×10^{10} pfu/ml. In addition to confirming the randomness of the library, we also validated our biopanning approach using thrombin as a test enzyme with previously known substrate specificity. Our results obtained with thrombin as a validating proteinase (data not shown here) matched with the known target specificity for this coagulation proteinase, especially in terms of a preference for arginine/lysine at the P1 position, proline, glycine, serine at P2, valine, glycine at P3, threonine, alanine, glycine at P1′ and glycine at the P2′ position.

Titration of phage-displayed library

The samples of phage plaques obtained after every round of biopanning were serially diluted and plated onto LB (Amp^+) Agar medium after mixing with suspension of *E. coli* (BLT5403) culture in 0.1 M $MgSO_4$ and top agarose. The phage plaques growing on the plates were manually counted after incubating the plates for 3–4 h at 37°C.

Biopanning of phage-displayed library with proteases and selection

Biopanning was performed using either 2.0 units/ml of ISP enzyme complex (Sharma et al., 2006) per cycle or 3.0 units/ml of purified KLK6 (Oikonomopoulou et al., 2006a, 2008) in 25 mM Tris-Cl, pH 8.0 (total volume of reaction mix: 0.5 ml) at 37°C overnight, subsequent to the binding of the amplified phage preparation (1×10^{10} pfu) with 100 μl Ni-NTA agarose beads as described in Karlson et al. (2002). A control elution was performed using 500 mM Imidazole solution. The process was repeated five times and the phage plaques obtained were amplified by lysing cultures of *E. coli* (BLT 5403). Amplified phage plaques from round five of biopanning were plated out on a top agarose plate and were picked up for subsequent amplification of DNA by PCR (using T7 primers). The PCR product was purified using QIAquick PCR purification kit (Qiagen, Mississauga, ON, Canada) and sequenced. The amino acid sequence of the random peptides displayed is deduced from the DNA sequence thus obtained.

In vitro cleavage of synthetic peptide and identification by HPLC-mass spectrometric analysis

Synthetic peptide (1 mg/ml) diluted in 25 mM Tris-Cl (pH 7.8) was incubated with 3.0 units of purified recombinant KLK6 (total volume of reaction mixture: 0.5 ml) at 37°C overnight. The reaction mixture was separated using HPLC with an acetonitrile gradient (0–50% v/v over 30 min) in 0.1% v/v trifluoroacetic acid, using a flow rate of 1 ml/min. The positions of the hydrolysis products monitored by ultraviolet absorption (E_{215}) were compared to the elution position of the intact peptide. The masses of the individual peaks (E_{215}) were determined by MALDI-TOF mass spectroscopy. The masses were used to deduce the predicted amino acid sequences, based on the parent peptide sequence (amino acids are abbreviated by their one-letter

Table 5 Plaque sequences of random hexamer region obtained after selection of random phage plaques from the T7 library before biopanning.

Plaque no.	AA1	AA2	AA3	AA4	AA5	AA6
1	R	W	A	R	G	K
2	T	E	N	L	T	E
3	I	L	L	Y	N	R
4	R	V	S	Q	C	S
5	Y	S	F	E	R	K
6	G	A	L	W	T	H
7	G	R	L	M	S	R
8	T	F	N	A	Q	A
9	H	P	G	F	W	L
10	T	L	R	R	H	V
11	P	E	E	S	A	G
12	V	E	R	R	N	G
13	G	S	M	E	H	G
14	C	Q	V	P	P	R
15	P	R	R	M	K	W
16	C	A	M	M	P	R
17	P	G	G	H	V	R
18	E	R	M	E	R	R
19	G	T	M	G	V	T
20	G	A	K	R	E	I
21	R	F	V	P	L	R
22	C	V	R	C	H	D

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Table 6 Amino acid content in the random hexamer region of the randomly selected phage particles before biopanning.

Amino acid	Nominal frequency[a]	Expected	Observed	Observed/expected[a]
F	0.032	4	4	1
L	0.097	13	8	0.615
S	0.097	13	6	0.462
P	0.065	9	8	0.888
I	0.032	4	2	0.5
M	0.032	4	7	1.75
T	0.065	9	7	0.777
V	0.065	9	8	0.888
A	0.065	9	7	0.777
Y	0.032	4	2	0.5
C	0.032	4	5	1.25
W	0.032	4	4	1
H	0.032	4	6	1.50
Q	0.032	4	3	0.75
R	0.097	13	23	1.77
N	0.032	4	4	1
K	0.032	4	4	1
D	0.032	4	1	0.25
G	0.065	9	13	1.44
E	0.032	4	10	2.5

[a]Nominal frequency was obtained as described by Cwirla et al. (1990).
[b]The acceptable ratio of observed vs. expected frequency of occurrence ranges from 0.5 to 2.0 confirming the randomness of the library. The only minor anomalies observed are in the case of aspartic acid (D) and glutamate (E).

codes, e.g., A=alanine, R=arginine). Except where indicated, all chemical reagents were from VWR International (Mississauga, ON, Canada).

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