Biochemical and Biophysical Research Communications 433 (2013) 333–337

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Human tissue kallikreins 3 and 5 can act as plasminogen activator releasing active plasmin

Lucas R. de Souza [a,1], Pollyana M. Melo [b,1], Thaysa Paschoalin [b], Adriana K. Carmona [b], Marcia Kondo [b], Izaura Y. Hirata [b], Michael Blaber [c], Ivarne Tersariol [d], Joyce Takatsuka [e], Maria A. Juliano [b], Luiz Juliano [b], Roseli A. Gomes [e], Luciano Puzer [a,*]

[a] Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Santo André, SP, Brazil
[b] Departamento de Biofísica, Universidade Federal de São Paulo, SP, Brazil
[c] Department of Biomedical Sciences, Florida State University College of Medicine, Tallahassee, FL, USA
[d] Centro Interdisciplinar de Investigação Bioquímica, Universidade de Mogi das Cruzes, SP, Brazil
[e] Instituto de Ciências Biológicas e Naturais, Universidade Federal do Triangulo Mineiro, Uberaba, MG, Brazil

ARTICLE INFO

Article history:
Received 23 February 2013
Available online 13 March 2013

Keywords:
Plasminogen
Plasmin
Kallikrein
Protease

ABSTRACT

Human tissue kallikreins (KLKs) are a group of serine proteases found in many tissues and biological fluids and are differentially expressed in several specific pathologies. Here, we present evidences of the ability of these enzymes to activate plasminogen. Kallikreins 3 and 5 were able to induce plasmin activity after hydrolyzing plasminogen, and we also verified that plasminogen activation was potentiated in the presence of glycosaminoglycans compared with plasminogen activation by tPA. This finding can shed new light on the plasminogen/plasmin system and its involvement in tumor metastasis, in which kallikreins appear to be upregulated.

© 2013 Elsevier Inc. Open access under the Elsevier OA license.

1. Introduction

Human tissue kallikreins (KLKs) are a group of secreted serine proteases found in diverse biological tissues and fluids and are differentially expressed in several pathological conditions [1]. This family of proteases is composed of 15 members with considerable similarities, and their genes are localized in tandem on chromosome 19q13.4 [1–4]. In the last decade, the number of studies regarding the kallikrein family has increased significantly, with many of the studies investigating the ability of KLKs to cleave extracellular matrix and plasma components, including collagens, fibrinogen, gelatin, laminin, and plasminogen. The ability of KLKs to degrade multiple components of the extracellular matrix can promote tumor growth, metastasis and invasion [5,6].

Plasminogen is a single-chain, multidomain glycoprotein of approximately 90 kDa, and it is composed of an N-terminal peptide, five disulfide-linked structures known as kringle domains, and the trypsin-like serine protease plasmin [7]. Plasminogen is expressed as a zymogen that can be processed to plasmin by two physiological activators, tissue plasminogen activator (tPA) [7] and urokinase type plasminogen activator (uPA) [8], which cleave the Arg561–Val562 peptide bond. Plasminogen is involved in controlling angiogenesis through its cleavage of peptides containing full or partial kringle domains, including the angiostatin-like peptides [9]. However, the primary role of plasminogen is in the coagulation cascade, where it is processed to plasmin, which acts in fibrinolysis to digest various clotting components.

Previous studies have revealed that plasmin either directly cleaves the extracellular matrix (ECM) [10] and basement membrane components or indirectly contributes to cleavage by activating other enzymes that degrade the structural components [11]. This indicates a role for plasmin in tumor progression and invasion and, consequently, metastasis. This hypothesis is reinforced by the fact that inhibiting various components involved in fibrinolysis blocks plasmin activation and decreases tumor cell migration and invasion [10,12]. Furthermore, over-expression of plasminogen/plasmin system members is observed in pancreatic cancer and is closely related to the dissociation of these aggressive cancer cells [11]. Here, we present a study on plasminogen activation by the KLKs in the hopes of shedding new light on the plasminogen/plasmin system and its involvement in tumor metastasis, mainly in which kallikreins appear to be upregulated.

2. Materials and methods

2.1. Enzymes, substrates and buffers

Mature human tissue kallikreins were expressed as recombinant proteins from an insect cell/baculovirus expression system,

* Corresponding author.
E-mail address: luciano.puzer@ufabc.edu.br (L. Puzer).
[1] These authors contributed equally for the results.

0006-291X © 2013 Elsevier Inc. Open access under the Elsevier OA license.
http://dx.doi.org/10.1016/j.bbrc.2013.03.001

as previously described [13]. Plasminogen was obtained from Calbiochem (San Diego, CA, USA). The plasmin substrate S2251 was obtained from Chromogenix (Milano, Italy).

2.2. Peptide synthesis

An automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu) was used for the synthesis of FRET (fluorescence resonance energy transfer) peptides using the Fmoc procedure with NovaSyn TGR resin (Novabiochem, San Diego, CA, USA) [14].

2.3. Plasminogen cleavage assay

Human plasminogen (44.5 μM) was incubated with 4.5 μM recombinant human tissue kallikrein 1, 3, 5, and 7 in 50 mM Tris–HCl buffer, pH 7.5, containing 1.0 mM EDTA and 0.01% Tween-20 at 37 °C for 24 h. The control assays were performed without enzyme. The incubations were quenched by adding SDS sample buffer containing 100 mM dithiothreitol and boiled for 5 min. The samples were run on 10% SDS polyacrylamide gels, and the protein fragments were visualized after staining with Coomassie brilliant blue R-250. The scanned images were stored as TIFF files.

2.4. N-terminal sequencing of plasminogen fragments

The fragments resulting from plasminogen digestion by kallikreins were fractionated on 10% SDS–PAGE as described above and electrotransferred onto methanol-activated polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA) in glycine-containing transfer buffer. Glycine was removed from the membrane by rinsing three times for 10 min each with 100 ml of ultra-pure water. Protein bands were stained for 10 min with 0.025% Coomassie brilliant blue R-250. The bands were excised from the membranes, and the N-terminal amino acid sequence was analyzed with a PPSQ/23 amino acid sequencer (Shimadzu Corporation, Tokyo, Japan).

2.5. Plasmin activity assay

Plasmin activity was measured in microassay plates using 0.055 μM plasminogen and 0.5 μM KLK1, KLK3, KLK5 or KLK7. Incubations were carried out in the presence of 200 μM of the chromogenic substrate D-Val-Leu-Lys-*p*-nitroanilide (S2251). The assays were performed in 50 mM Tris buffer, pH 7.5, at 37 °C for 1, 4 or 24 h. As a positive control, 0.055 μM of plasminogen and 0.5 μM of tissue plasminogen activator (tPA) were combined in the presence of the substrate D-Val-Leu-Lys-*p*-nitroanilide under the same conditions. A negative control was evaluated using only kallikreins or plasminogen in the presence of the chromogenic substrate. The activity of plasmin against the chromogenic substrate was evaluated by measuring the absorbance at 405 nm. The influence of GAGs (heparin, dermatan sulfate and chondroitin sulfate, 10 μM of each) and sodium citrate (1 M) on kallikrein 3 and kallikrein 5 activity was also tested under the same experimental conditions.

2.6. Statistical analysis

Statistical analysis was carried out with the Prism software package. Statistical significance was determined using Student's *t*-test and the Bonferroni test. The results were considered to be significant at $P < 0.05$.

3. Results and discussion

To better understand the specificity of the human tissue kallikreins, we initiated a study to evaluate the degradation pattern of various proteins incubated with KLKs. The degradation of plasminogen by KLKs drew our attention due to the persistent formation of a protein fragment of approximately 29 kDa resulting from proteolysis with only KLK3 and KLK5, and this fragment had not yet been reported in other similar studies [5,6]. Human plasminogen was incubated with recombinant KLK1, KLK3, KLK5, or KLK7 as described, and Fig. 1A shows the fragments of degraded plasminogen

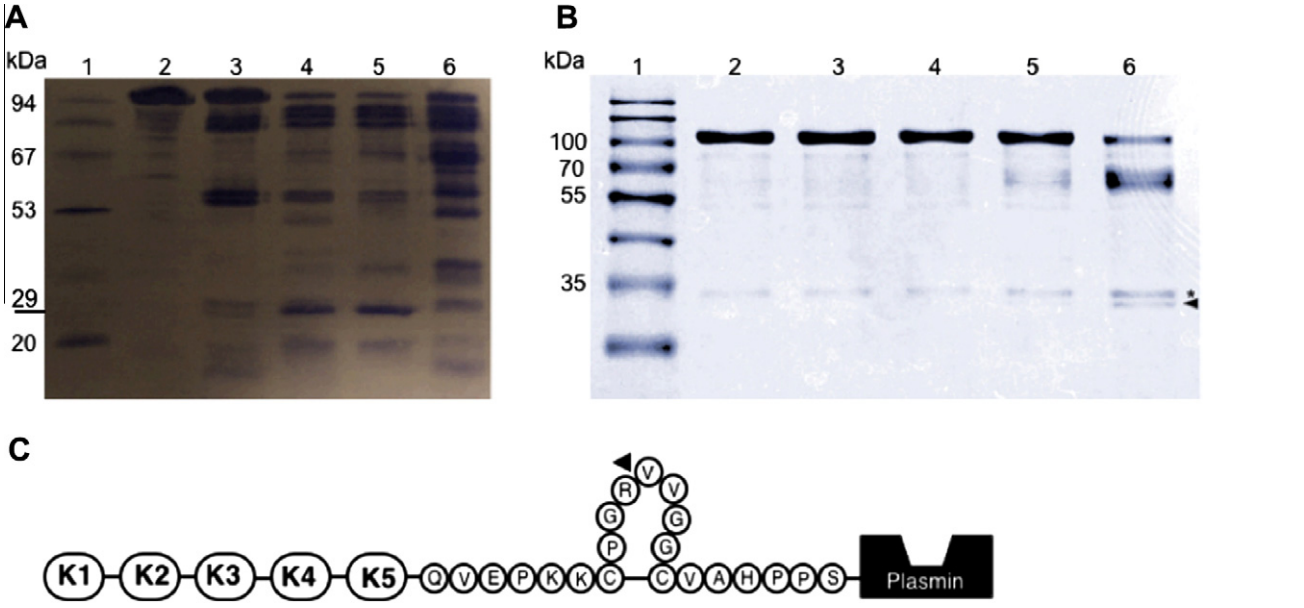

Fig. 1. Hydrolysis of plasminogen by human tissue kallikreins. (A) Lane 1, molecular weight standard; lane 2, plasminogen; lanes 3, 4, 5 and 6, plasminogen incubated with KLK1, KLK3, KLK5 and KLK7, respectively. The N-terminal sequence of the 29 kDa bands in lanes 4 and 5 was determined through Edman degradation. (B) Hydrolysis of plasminogen by human tissue kallikrein 3. Lane 1, molecular weight standard; lane 2, plasminogen incubated with preheated KLK3; lanes 3, 4, 5 and 6, plasminogen incubated with KLK3 for 1, 4, 10 and 24 h, respectively. The * indicates the KLK3 band; the (◀) indicates the plasmin band. (C) Schematic representation of the plasminogen sequence. The five kringle domains, the site of plasminogen activation by tPA (♦), and the active plasmin location.

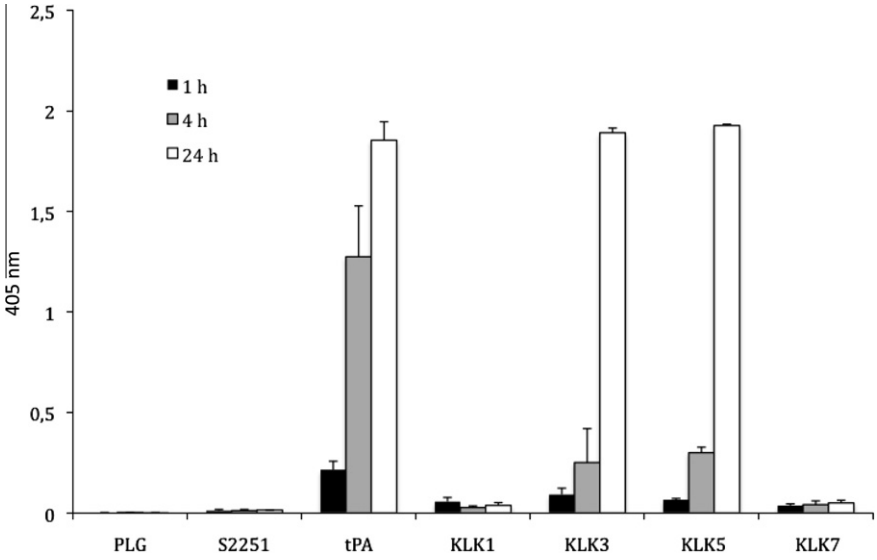

Fig. 2. Activity of plasmin determined by hydrolysis of the substrate S2251. The kallikreins were incubated with plasminogen and the substrate S2251, and the absorbance of the substrate was monitored at 405 nm for 1, 4 and 24 h. The experimental conditions were described in the Section 2.

that were generated after 24 h of incubation. KLK3 and KLK5 presented almost the same proteolysis pattern, and the incubation of plasminogen with both enzymes generated a fragment of approximately 29 kDa, which was not observed in the incubation with KLK1 and KLK7. The proteolysis profile of KLK3 and KLK5 against plasminogen was better demonstrated by a time course experiment (Fig. 1B), which showed that the 29 kDa fragment appeared at 10 h of incubation. After transferring the 29 kDa fragment to a PVDF membrane, the N-terminal sequence was identified to contain H_2N-Val-Val-Gly-Gly-Xaa-Val-Ala. We were not able to identify the amino acid cysteine, which was represented by Xaa.

As previously discussed, the activation of plasminogen to plasmin occurs by cleaving the Arg-Val peptide bond (Fig. 1C) at the site of a loop formed by a disulfide bridge. The N-terminal sequence of the 29 kDa fragment had an identical sequence to the one that would have been generated following plasminogen activation. To verify whether the 29 kDa fragment contained plasmin activity, each KLK was incubated with plasminogen in the presence of the specific plasmin substrate S2251. Fig. 2 shows the progressive hydrolysis of the S2251 substrate, as determined by monitoring the absorbance at 405 nm for 1, 4 and 24 h. The positive control was plasminogen in the presence of tissue plasminogen activator (tPA). The negative control used plasminogen and the S2251 substrate but omitted the KLKs. Fig. 2 reveals the difference in the absorbance of the cleaved substrate S2251 in the presence of either the plasminogen/KLKs or the KLKs alone. An increase in absorbance was observed after 4 h of incubation with KLK3 and KLK5 but not KLK1 and KLK7. Compared to the positive control, KLK3 and KLK5 were approximately 20% as efficient as tPA in activating plasminogen at 4 h. However, at 24 h of incubation, both KLK3 and KLK5 showed similar activity to tPA by completely hydrolyzing the S2251 substrate. In contrast, KLK1 and KLK7 did not produce any detectable increases in absorbance even after 24 h, indicating that the S2251 substrate was not cleaved.

There have been several reports discussing the hydrolysis of plasminogen by kallikreins, indicating that these enzymes are capable of generating angiostatin-like molecules [5]. However, these studies did not discuss the ability of KLKs to generate the active enzyme plasmin via plasminogen hydrolysis. Michael and colleagues [5] showed the capacity of KLK5 to hydrolyze plasminogen at two different peptide bonds, Lys77-Lys78 and Arg549-Lys550, releasing two fragments that function as angiostatin-like molecules. In their study, plasminogen hydrolysis was monitored for 8 h by SDS–PAGE, and they did not detect any other fragments that were similar in size to plasmin. We believe that the amount of plasminogen (0.5 μg) and the incubation time course (8 h) employed by Michael and colleagues were insufficient to generate a detectable amount of plasmin-related fragments for visualization by SDS–PAGE. In another publication, Heidtmann and colleagues [6] incubated 60 μg of plasminogen with 3 μg of human KLK3 for 6 h, and they also failed to detect any fragments related to plasmin. In a similar experiment, we used 60 μg of plasminogen, and after 10 h, it was possible to visualize a 29 kDa protein fragment whose N-terminal sequence was similar to the N-terminus of plasmin.

KLKs were also verified to exhibit low activity against the S2251 substrate, which is largely used for monitoring plasmin cleavage. KLKs weakly hydrolyze the S2251 substrate and, after 24 h of incubation, the change in absorbance indicated that the addition of KLK3 and KLK5 did not significantly alter the substrate, and the final absorbance was similar to the intrinsic absorbance of the substrate.

As previously mentioned, because glycosaminoglycans (GAGs) and sodium citrate improve KLK3 [15] activity, we assayed the influence of heparin, dermatan sulfate and chondroitin sulfate on plasminogen activation by KLK3 and KLK5. Fig. 3 shows the progression of S2251 substrate hydrolysis through monitoring the absorbance at 405 nm for 1 and 4 h following the incubation of plasminogen with KLKs in the presence of GAGs. Notably, both KLK3 and KLK5 activated plasminogen with nearly the same potency as tPA in the presence of heparin. Furthermore, KLK3 appeared to be more potent than tPA in activating plasminogen in the presence of dermatan sulfate. In contrast, the addition of sodium citrate did not produce any noticeable improvement in plasminogen activation by KLKs. The ability of GAGs to improve plasmin formation has also been described for the activation of plasminogen by tPA [16].

GAGs can interact with hundreds of proteins, regulating multiple signaling pathways. In contrast, abnormal protein/GAG aggregates are associated with a variety of pathological conditions, including Alzheimer's, diabetes and different types of cancers [17]. Thus, the ability of GAGs to enhance KLK3 and KLK5 proteolysis against plasminogen, releasing active plasmin, can have implications for cancer development, as both enzymes appear to be upregulated in some types of cancers, mainly prostate and ovarian cancers [18–19].

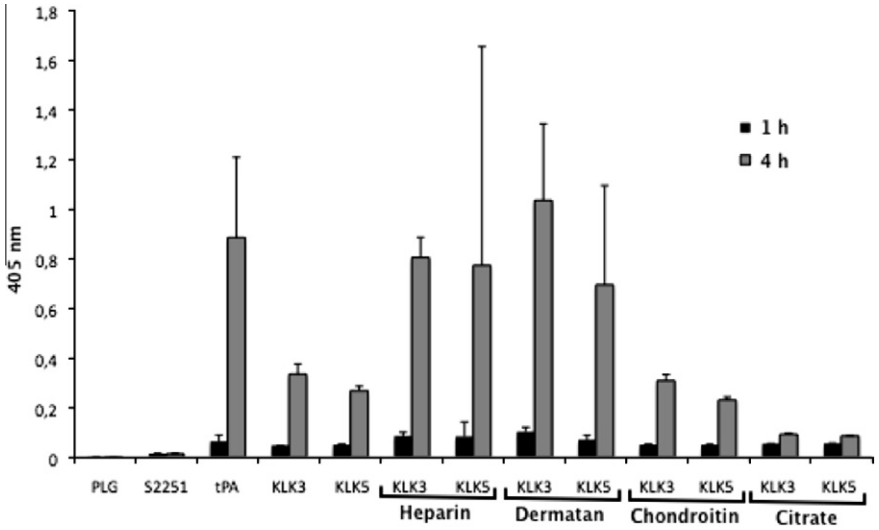

Fig. 3. Plasmin activity determined by hydrolysis of the substrate S2251 in the presence of heparin, dermatan sulfate, chondroitin sulfate and sodium citrate. KLK3 and KLK5 were incubated with plasminogen in the presence of substrate S2251 and the indicated glycosaminoglycan. The absorbance of the substrate was monitored at 405 nm for 1, 4 and 24 h. The experimental conditions were described in the Section 2.

It is also noteworthy that KLK3 is typically described as a protease with chymotrypsin-like specificity and thus most efficiently hydrolyzes substrates with a hydrophobic residue at the P1 position, manly tyrosine and phenylalanine. However, N-terminal sequencing revealed that KLK3 activated plasminogen by cleaving the peptide bond following an arginine residue. Therefore, we synthesized the peptides Abz-APGRVVGGAQ-EDDnp, H_2N-CPGRVVGGC-NH_2 and H_2N-QVEPKKCPGRVVGGCVAHPHS-NH_2, which comprise the loop leading to the active site of plasminogen (Fig. 1C). This experiment was performed to determine the kinetic parameters of the hydrolysis. The first peptide was prepared with two alanines substituting for the cysteine residues, and the other two peptides were prepared to form the disulfide bridge that serves as the loop that is cleaved for plasminogen activation. However, no KLK3 or KLK5 proteolytic activity was detected against the two peptides with the disulfide bridge, even in the presence of GAGs or sodium citrate. Only the peptide that contained the two alanines substituting for the two residues of cysteine was hydrolyzed by both KLK3 and KLK5 (Fig. 4). In all assays, KLK5 activity was higher than KLK3 activity. However, due to the low activity detected, it was not possible to determine the kinetic parameters K_m and V_{max}, even in the presence of GAGs and sodium citrate. These observations suggest a more complex interaction between GAGs, KLKs and the substrate (plasminogen or synthetic peptide) and indicate that the enhanced activity of KLK3 and KLK5 in the presence of GAGs is also dependent on the target substrate.

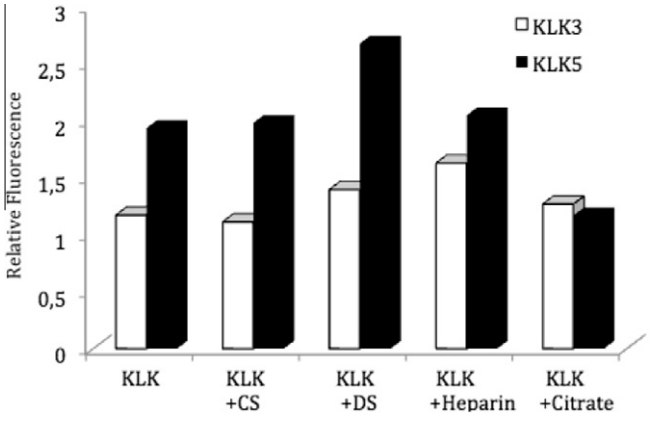

Fig. 4. Activity of KLK5 and KLK7 against the quenched fluorescent peptide Abz-APGRVVGGA-EDDnp. The enzyme activities were also assayed in the presence of GAGs and sodium citrate.

In conclusion, here we presented evidences that indicate the capacity of KLK3 and KLK5 to activate plasminogen. As both KLKs are expressed in several tissues and secreted into the circulation and other major biological fluids [3], the ability of these enzymes to activate plasminogen could represent a new way to understand their involvement in various pathological conditions, primarily cancer and inflammation processes, in which KLKs appear to be upregulated.

Acknowledgments

We gratefully acknowledge financial support from FAPESP (Fundação de Amparo à Pesquisa do Estado de São Paulo, Proc 2011/51297-8), CNPq (Conselho Nacional de Pesquisa e Desenvolvimento, Proc. 312701/2009-8), and FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais).

References

[1] G.M. Yousef, E.P. Diamandis, The new human tissue kallikrein gene family: structure, function, and association to disease, Endocr. Rev. 22 (2001) 184–204.
[2] G. Pampalakis, G. Sorotipoulou, Tissue kallikrein proteolytic cascade pathways in normal physiology and cancer, Biochim. Biophys. Acta 1776 (2007) 22–31.
[3] C.A. Borgono, E.P. Diamandis, The emerging roles of human tissue kallikreins in cancer, Nat. Rev. Cancer 4 (2004) 876–890.
[4] L. Luo, W. Jiang, Inhibition profiles of human tissue kallikreins by serine protease inhibitors, Biol. Chem. 387 (2006) 813–816.
[5] I.P. Michael, G. Sotiropoulou, G. Pampalakis, A. Magklara, M. Ghosh, G. Wasney, E.P. Diamandis, Biochemical and enzymatic characterization of human kallikrein 5 (hK5), a novel serine protease potentially involved in cancer progression, J. Biol. Chem. 280 (2005) 14628–14635.
[6] H.H. Heidtmann, D.M. Nettelbeck, A. Mingels, R. Jäger, H.G. Welker, R.E. Kontermann, Generation of angiostatin-like fragments from plasminogen by prostate-specific antigen, Br. J. Cancer 81 (1999) 1269–1273.
[7] T.E. Petersen, M.Q. Martzen, A. Ichinose, E.W. Davie, Characterization of the gene for human plasminogen, a key proenzyme in the fibrinolytic system, J. Biol. Chem. 265 (1990) 6104–6111.
[8] G. Frenette, R.R. Tremblay, C. Lazure, J.Y. Dube, Prostatic kallikrein hk2, but not prostate-specific antigen (hk3), activates single-chain urokinase-type plasminogen activator, Int. J. Cancer 71 (1997) 897–899.
[9] A. Galaup, C. Magnon, V. Rouffiac, P. Opolon, D. Opolon, N. Lassau, T. Tursz, M. Perricaudet, F. Griscelli, Full kringles of plasminogen (aa 1–566) mediate complete regression of human MDA-MB-231 breast tumor xenografted in nude mice, Gene Ther. 12 (2005) 831–842.

[10] G. Huang, Z. Hu, M. Li, Y. Cui, Y. Li, L. Guo, W. Jiang, S.H. Lu, ECRG2 inhibits cancer cell migration, invasion and metastasis through the downregulation of uPA/plasmin activity, Carcinogenesis 28 (2007) 2274–2281.
[11] X. Tan, H. Egami, F. Nozawa, M. Abe, H. Baba, Analysis of the invasion-metastasis mechanism in pancreatic cancer: involvement of plasmin(ogen) cascade proteins in the invasion of pancreatic cancer cells, Int. J. Oncol. 28 (2006) 369–374.
[12] H. Kobayashi, H. Shinohara, K. Takeuchi, Inhibition of the soluble and the tumor cell receptor-bound plasmin by urinary trypsin inhibitor and subsequent effects on tumor cell invasion and metastasis, Cancer Res. 54 (1994) 844–849.
[13] T.S.P. Teixeira, R.F. Freitas, O. Abraha~o Jr, K.F. Devienne, L.R. de Souza, S.I. Blaber, M. Blaber, M.Y. Kondo, M.A. Juliano, L. Juliano, L. Puzer, Biological evaluation and docking studies of natural isocoumarins as inhibitors for human kallikrein 5 and 7, Bioorg. Med. Chem. Lett. 21 (2011) 6112–6115.
[14] I.Y. Hirata, M.H.C. Cezari, C.R. Nakaie, P. Boshcov, A.S. Ito, M.A. Juliano, L. Juliano, Internally quenched fluorogenic protease substrates: solid-phase synthesis and fluorescent spectroscopy of peptides containing ortho-aminobenzoil/dinitrophenyl groups as donor–acceptor pairs, Lett. Pept. Sci. 1 (1995) 299–308.
[15] D. Andrade, D.M. Assis, A.R. Lima, J.R. Oliveira, M.S. Araujo, S.I. Blaber, M. Blaber, M.A. Juliano, L. Juliano, Substrate specificity and inhibition of human kallikrein-related peptidase 3 (KLK3 or PSA) activated with sodium citrate and glycosaminoglycans, Arch. Biochem. Biophys. 498 (2010) 74–82.
[16] P. Andrade-Gordon, S. Strickland, Interaction of heparin with plasminogen activators and plasminogen: effects on the activation of plasminogen, Biochemistry 25 (1986) 4033–4040.
[17] L. Zhang, Glycosaminoglycan (GAG) biosynthesis and GAG-binding proteins, Prog. Mol. Biol. Transl. Sci. 93 (2010) 1–17.
[18] N. Emami, E.P. Diamandis, Utility of kallikrein-related peptidases (KLKs) as cancer biomarkers, Clin. Chem. 54 (2008) 1600–1607.
[19] M. Avgeris, K. Mavridis, A. Scorilas, Kallikrein-related peptidases in prostate, breast, and ovarian cancers: from pathobiology to clinical relevance, Biol. Chem. 393 (2012) 301–317.