

Immunofluorometric activity-based probe analysis of active KLK6 in biological fluids

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Abstract

Immunoassay measurements of human kallikrein-related peptidases (KLKs) such as prostate-specific antigen (KLK3) are of great value as diagnostic indices of cancer. Despite extensive knowledge of the abundance of immunoreactive KLKs in normal and cancer-related settings, there is little information available about the proportion of immunoreactive KLK that represents active enzyme in such samples. Using KLK6 as a prototype enzyme, we have developed an assay using a serine proteinase-targeted activity-based probe coupled to antibody capture. By employing activity-based labeling, we were able to quantify the proportion of enzymatically active relative to total immunoreactive KLK6 in crude cerebrospinal fluid from routine analyses and ascites fluid from ovarian cancer patients, as well as in supernatants from cancer cell lines. Our approach allowed monitoring of pro-KLK6 conversion to its active enzyme species and demonstrated that up to 5% of immunoreactive KLK6 detected in clinical samples represents active enzyme. We suggest that this new activity-based probe assay will prove of value as a complement to routine KLK immunoassay measurements for validating KLKs as cancer biomarkers.

Keywords: activity-based probes; ascites fluid; cerebrospinal fluid; human kallikrein-related peptidases; serine proteinases; trypsin-like activity.

Introduction

Kallikrein-related peptidases are a family of serine proteinases sharing approximately 50% homology at the gene and protein level (Diamandis et al., 2000a; Borgeño and Diamandis, 2004; Borgeño et al., 2004). These enzymes are secreted as inactive zymogens and upon activation display trypsin- and/or chymotrypsin-like activity. One member of this family, kallikrein-related pep-

tidase 6 (now designated KLK6), was originally cloned by three different groups from breast (Anisowicz et al., 1996), brain (Little et al., 1997) and from a colon carcinoma cell line (Yamashiro et al., 1997). Not known at that time to represent the same enzyme, these proteinases were given the names protease M, zyme and neurosin, respectively. The rat ortholog of KLK6 is known as myelencephalon-specific protease (MSP; Scarisbrick et al., 1997; Blaber et al., 2002; Scarisbrick et al., 2002). Mouse orthologs of human KLK6 have also been identified as brain and skin protease (BSSP) (Meier et al., 1999) and brain serine protease (BSP) (Matsui et al., 2000).

Like the most widely recognized member of the KLK family, KLK3 or prostate-specific antigen, which is a valuable prostate cancer biomarker, KLK6 levels are now believed to represent a useful tumor biomarker, particularly in the setting of ovarian cancer (Borgono and Diamandis, 2004; Borgono et al., 2004). A role for KLK6 has also been suggested in the context of degenerative diseases of the central nervous system, including multiple sclerosis (Scarisbrick et al., 2002; Blaber et al., 2004), Alzheimer's disease (Diamandis et al., 2000b; Ogawa et al., 2000; Shimizu-Okabe et al., 2001; Mitsui et al., 2002; Zarghooni et al., 2002) and Parkinson's disease (Ogawa et al., 2000; Iwata et al., 2003). Apart from the central nervous system, a wide pattern of mRNA and protein expression for KLK6 has been documented throughout the human body (Yousef et al., 1999, 2005; Petraki et al., 2001, 2002; Shaw and Diamandis, 2007). Furthermore, immunoreactive KLK6 has been found in a variety of body fluids (Shaw and Diamandis, 2007), including breast milk and breast cyst fluid (Diamandis et al., 2000c), cerebrospinal fluid (CSF; Diamandis et al., 2000c; Okui et al., 2001), vaginal fluid (Shaw et al., 2007), and nipple aspirate fluid (Diamandis et al., 2000c; Sauter et al., 2004). The protein has also been detected in male and female serum, seminal plasma, and in a relatively small percentage of amniotic fluid samples and breast tumor cytosolic extracts (Diamandis et al., 2000c).

Although the specific mechanism(s) of activation of the KLK family *in vivo* are not yet clear, the regulation of their activity is achieved mainly through proteolytic processes of activation and inactivation, as well as by binding to inhibitors (Borgono and Diamandis, 2004; Borgeño et al., 2004). KLK6 is secreted as a zymogen and is activated by the removal of five amino acids from its N-terminal sequence (Gomis-Ruth et al., 2002). It has been reported that KLK6 can auto-catalytically activate via a two-step process, in which an internal bond in the activation peptide is recognized and cleaved first, followed by removal of a dipeptide to yield the active enzyme (Bayes et al., 2004). A recent study showed that the rate of this auto-activation process for KLK6 is slower than the enzyme's rate of auto-degradation (Blaber et al., 2007). At least one

of the intrinsic cleavage sites that can lead to protein degradation and inactivation has been identified (Bernett et al., 2002; Magklara et al., 2003; Bayes et al., 2004). The activity of KLK6 can be regulated not only by proteolytic processing of the active enzyme, but also by inhibitory serine proteinase inhibitors (serpins; Luo and Jiang, 2006), such as α 2-antiplasmin (Magklara et al., 2003), α 1-antichymotrypsin (Hutchinson et al., 2003) and antithrombin III (Magklara et al., 2003).

Although evaluated for the isolated enzyme *in vitro* (Bernett et al., 2002; Magklara et al., 2003; Bayes et al., 2004; Blaber et al., 2007), this activation and inactivation process has yet to be monitored *in vivo*. Furthermore, in spite of the extensive literature dealing with the abundance of immunoreactive KLKs in a variety of normal and cancer-related settings, there is as yet little information available on the proportion of immunoreactive KLK that represents active enzyme in such samples.

The mechanism whereby enzymatically active KLKs, including KLK6, may regulate tissue function *in vivo* is the subject of much recent interest (Hansen et al., 2007; Hollenberg et al., 2007). Some effects of KLKs in the context of cancer progression are believed to be due to the cleavage of a number of extracellular targets, such as proteins of the extracellular matrix, pro-urokinase-plasminogen activator, kininogens, growth factor precursors (and binding proteins) and other KLKs (Borgoño and Diamandis, 2004). In addition, our own work has revealed that the KLKs, including KLK6, can signal to cells via proteinase-activated receptors (PARs; Oikonomopoulou et al., 2006a,b,c). Activation of these G protein-coupled receptors can initiate multiple signaling pathways related to inflammation, carcinogenesis, invasion, metastasis, angiogenesis, and cell death and survival (Ossovskaya and Bunnett, 2004; Steinhoff et al., 2005; Hansen et al., 2007; Ramachandran and Hollenberg, 2008). Thus, we suggest that an understanding of the pathophysiological role of KLKs *in vivo* will require measurements not only of the total immunoreactive levels of enzyme that may be present, but also of the proportion of enzyme that is present in its catalytically active state, which can regulate tissue signaling by both PAR-dependent and PAR-independent mechanisms.

To monitor the presence of enzymatically active serine proteinases such as the KLKs in cell or tissue-derived samples, we have used activity-based probes (ABPs), which irreversibly modify the enzyme active-site nucleophile and, by virtue of a reporter tag, allow the detection and purification of individual labeled enzymes through simple separation techniques such as SDS-PAGE (Baruch et al., 2004; Hansen et al., 2005; Pan et al., 2006; Sadaghiani et al., 2007). Using KLK6 as a prototype member of the KLK family with serine proteinase activity, we sought to develop an analytical approach using a serine proteinase-targeted ABP coupled to immunoassay measurements for the following purposes: (1) to monitor the conversion of KLK6 from its zymogen to its enzymatically active species; (2) to develop an improved procedure for the isolation of active KLK (free from its zymogen) to facilitate studies of the active enzyme; and (3) using a new ratiometric assay, to quantify the proportion of enzymatically active KLK6 (relative to total

immunoreactive KLK6) present in ovarian cancer-derived ascites fluid, CSF and supernatants from cancer-related cell cultures.

Results

Production and purification of KLK6 zymogen and monitoring of its activation by thermolysin

KLK6 zymogen, with minimal detectable enzyme activity against *t*-butoxycarbonyl-Val-Pro-Arg-7-amino-4-methylcoumarin (VPR-AMC), was isolated chromatographically as previously described (Magklara et al., 2003) and the recombinant protein was activated with thermolysin. Control samples lacking thermolysin were incubated under the same conditions. After 2 h of incubation, more than 99% of the KLK6 zymogen (Figure 1, upper panel, solid arrowhead) was converted to a form of lower molecular weight and greater mobility on SDS-PAGE, as detected by Coomassie staining (Figure 1, upper panel, open arrowhead). Within the same time frame, we found no evidence of further cleavage of the enzyme (due to non-specific proteolytic activity of thermolysin or to KLK6 auto-degradation) according to electrophoretic analysis of the protein with either Coomassie (Figure 1, upper panel) or silver staining (data not shown). Once activated, the enzyme activity of the KLK6 preparation was stable on storage in 50 mM sodium acetate buffer, pH 5.3 (retention of >90% KLK6 activity for close to 60 h at room temperature; data not shown). Western blot analysis of the same samples revealed faint bands of lower molecular mass at about 26 kDa, possibly representing KLK6 degradation fragments (Figure 1, middle panel). These minor fragments that may have been generated during thermolysin activation were not labeled by the activity-based probe (ABP) (Figure 1, compare middle and lower panels) and therefore represent immunoreactive enzymatically inactive fragments. Such inactive degradation products have also been reported in the literature (Bernett et al., 2002; Magklara et al., 2003; Bayes et al., 2004). Activity-based labeling using the biotin-linker-Pro-Lys-diphenylphosphonate probe (Bio-PK) revealed very rapid processing of the zymogen, with increasing levels of catalytically active KLK6 appearing over the 2-h time frame of incubation (Figure 1, left part of lower panel). A very low but detectable level of residual enzyme activity was also found in the samples that were not treated with thermolysin (Figure 1, right part of lower panel).

Production and purification of active KLK6

We exploited the ability of thermolysin to activate KLK6 rapidly and efficiently to develop a method of enzyme purification and activation directly from the KLK6-expressing HEK-293 cell supernatants. The method included two rounds of ion-exchange liquid chromatography purification (FPLC), separated by the activation step, as described in detail in the Materials and methods section. A representative second-round chromatogram of FPLC with and without the KLK6 activation step by thermolysin is shown in Figure 2A. FPLC fractions were reacted with the Bio-PK probe, resolved by SDS-PAGE

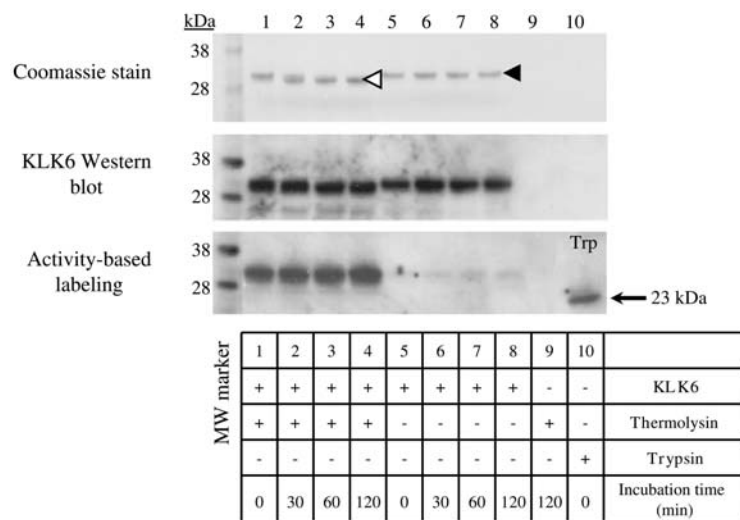


Figure 1 Monitoring activation of recombinant KLK6 by thermolysin.

Activation of the zymogen was achieved as outlined in the text over a 2-h time period, with termination of the reaction by addition of 50 mM EDTA. The reaction proceeded without the appearance of KLK6 degradation fragments (silver stain detection; data not shown). Conversion of the zymogen to active enzyme was monitored by SDS-PAGE analysis followed by detection of KLK6 by: (1) upper panel, Coomassie stain; (2) middle panel, Western blotting; and (3) lower panel, activity-based probe labeling followed by streptavidin-alkaline phosphatase visualization. The 23-kDa band in the bottom gel shows the activity-based probe labeling of 100 ng of porcine trypsin (Trp). The table below the gel images provides details for each lane designated by the numbers at the top of the gels. Active KLK6 (open arrowhead, upper panel) migrated more rapidly in the gel than the higher-molecular-mass zymogen (solid arrowhead, upper panel). Thermolysin treatment rapidly activated the enzyme within the timeframe of sample preparation (lane 1, lower panel), as shown by the reaction with biotinylated activity-based probe visualized using streptavidin-alkaline phosphatase. The ABP reacts only with active KLK6.

under reducing conditions and detected by streptavidin-based Western blotting to detect serine proteinases that had reacted with the biotinylated ABP (Figure 2B, lower panel, open arrowheads). This analysis proved that the FPLC-thermolysin activation-FPLC method can yield high levels of pure and active KLK6, which was visualized separately as the major component detected by Coomassie staining (Figure 2B, upper panel).

Quantification of KLK6 in biological fluids

The general concept of the KLK6 ABP ratiometric ELISA (KLK6 ABRA-ELISA) depends on quantification of active KLK6, which is described in Figure 3A. The first step involves reaction of Bio-PK with active KLK6. To immobilize the probe-KLK6 complex, we used a KLK6-targeted monoclonal antibody that has been previously generated and validated for its sensitivity and KLK6 selectivity in our laboratory (Luo et al., 2006). Detection of the probe-biotin label covalently linked to KLK6 was performed similarly to the regular KLK6 ELISA assay, incorporating streptavidin-linked detection reagents. In brief, we added a streptavidin-alkaline phosphatase (SA-ALP) conjugate, incubated, washed and then detected the ALP activity using diflunisal phosphate as substrate, in combination with terbium chelates and time-resolved fluorescence (Christopoulos and Diamandis, 1992; Ferguson et al., 1996; Diamandis et al., 2000c). This assay configuration demonstrated good sensitivity and specificity to quantify active KLK6. Optimal conditions were selected based on the lowest achievable detection limit and best assay linearity and dynamic range.

A typical calibration curve of the active KLK6 ELISA assay is shown in Figure 3B. The detection limit, defined

as the concentration of KLK6 corresponding to the fluorescence of the zero calibrator plus 2SD, was 0.025 $\mu\text{g/l}$ (~ 0.83 pM, considering a molecular mass of ~ 30 kDa revealed by SDS-PAGE of recombinant KLK6). Within- and between-run precision was assessed at various KLK6 concentrations between 0.05 and 10 $\mu\text{g/l}$ (1.67–333 pM) and with selected clinical samples. The coefficient of variation (CV) was between 6% and 20%.

To ensure that the assay specifically measures the active form of KLK6, we mixed inactive and active recombinant KLK6 in different ratios and analyzed the mixtures by ABRA-ELISA. The amount of active enzyme detected in these mixtures corresponded to the concentration of active KLK6 originally added to the mixture (data not shown). To confirm the specificity of the assay for active KLK6, we analyzed selected fluids for levels of active (ABP-reactive) and total (regular ELISA) KLK6: CSF from routine analyses, ascites fluid from ovarian cancer patients and cancer cell supernatants (Figure 4). Our analyses revealed that samples from the different sources had comparable levels of immunoreactive KLK6 (mean values ~ 70 – 270 $\mu\text{g/l}$ or ~ 2 – 9 nM). However, ABP-reactive enzyme levels were substantially lower and there was considerable heterogeneity in values obtained for the different samples, a number of which had ABP reactivity below our detection limit. For example, the mean concentration of active KLK6 was 0.33 $\mu\text{g/l}$ (11 pM) for CSF ($n=20$), 0.89 $\mu\text{g/l}$ (30 pM) for ascites fluid ($n=23$) and 0.029 $\mu\text{g/l}$ (0.97 pM) for cancer cell supernatants ($n=4$). The active KLK6 concentrations ranged from non-detectable to ~ 8 $\mu\text{g/l}$ (267 pM). Thus, we did not find a correlation between the amount of immunoreactive KLK6 and the levels of active enzyme. In summary, as a common feature of all samples, the majority of the immuno-

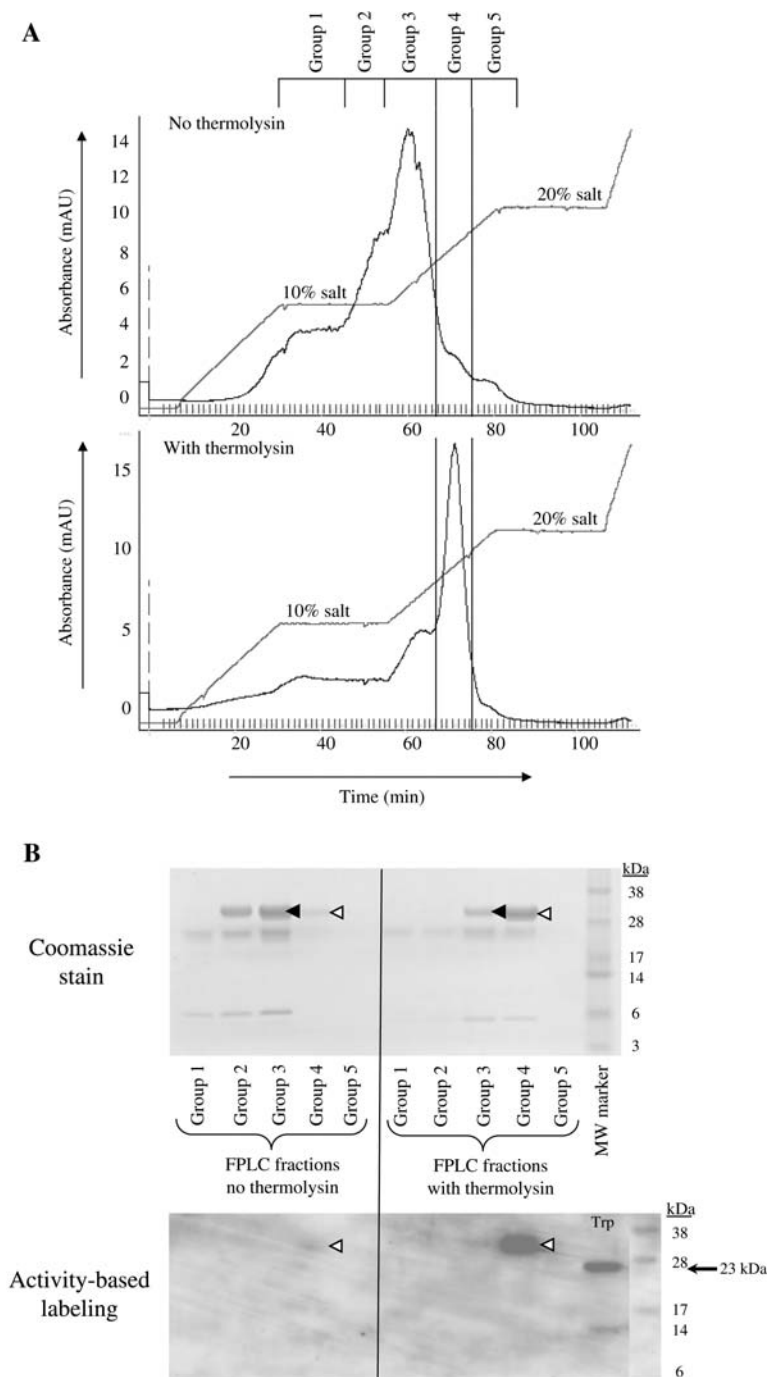


Figure 2 Production of active KLK6: FPLC chromatography of zymogen and thermolysin-activated enzyme (A) and SDS-PAGE analysis of protein and ABP-labeled enzyme (B).

(A) Zymogen preparation was harvested from cell supernatants by FPLC as outlined in Protocol B in the materials and methods section. Fractions containing KLK6 immunoreactivity (Western blot, data not shown) with no detectable enzyme activity (VPR-AMC analysis, data not shown) were pooled, treated or not with thermolysin and subjected to a second round of FPLC chromatography using a salt-step gradient, as also described under materials and methods (lines above/through the UV plots in chromatograms of panel A). The top chromatogram (A, upper) shows a representative FPLC chromatogram (UV absorbance at 280 nm) of a KLK6 zymogen preparation without prior activation with thermolysin. Fractions were monitored for KLK6 immunoreactivity and five groups of pooled samples were collected (Groups 1–5). The markings over the X-axis represent the FPLC fraction numbers. The lower chromatogram (A, lower) of thermolysin-activated KLK6 is the result of a salt-step gradient (shown as lines above/through the UV plots) that was the same as for the upper chromatogram. The fractions containing active enzyme, eluting between ~65 and 75 min (monitored by Western blotting and activity-based labeling; data not shown for individual fractions) were pooled (Group 4 pool). In total, five pooled fraction samples were obtained either with or without thermolysin activation, as shown by the vertical lines (Groups 1–5), and then analyzed further by SDS-PAGE (B) or incubated with the biotin-tagged ABP. Proteins in the gels were detected either by Coomassie Blue staining (upper gels) or using streptavidin-alkaline phosphatase reagent to visualize the biotin-tagged enzymes (lower gels), as outlined in the legend to Figure 1. The positions of the molecular mass markers (kDa) in each gel are shown on the right. The positions of active enzyme bands are denoted by open arrowheads (upper and lower gels); inactive zymogen is designated by the solid arrowhead (upper gels). Lower-molecular-mass bands visualized in the upper gels are likely the result of protein degradation, as discussed in the text. The 23-kDa band in the second-last lane from the right in the bottom gel shows the SDS-PAGE analysis of 100 ng of porcine trypsin (Trp) that had reacted with the biotin-tagged ABP (positive control).

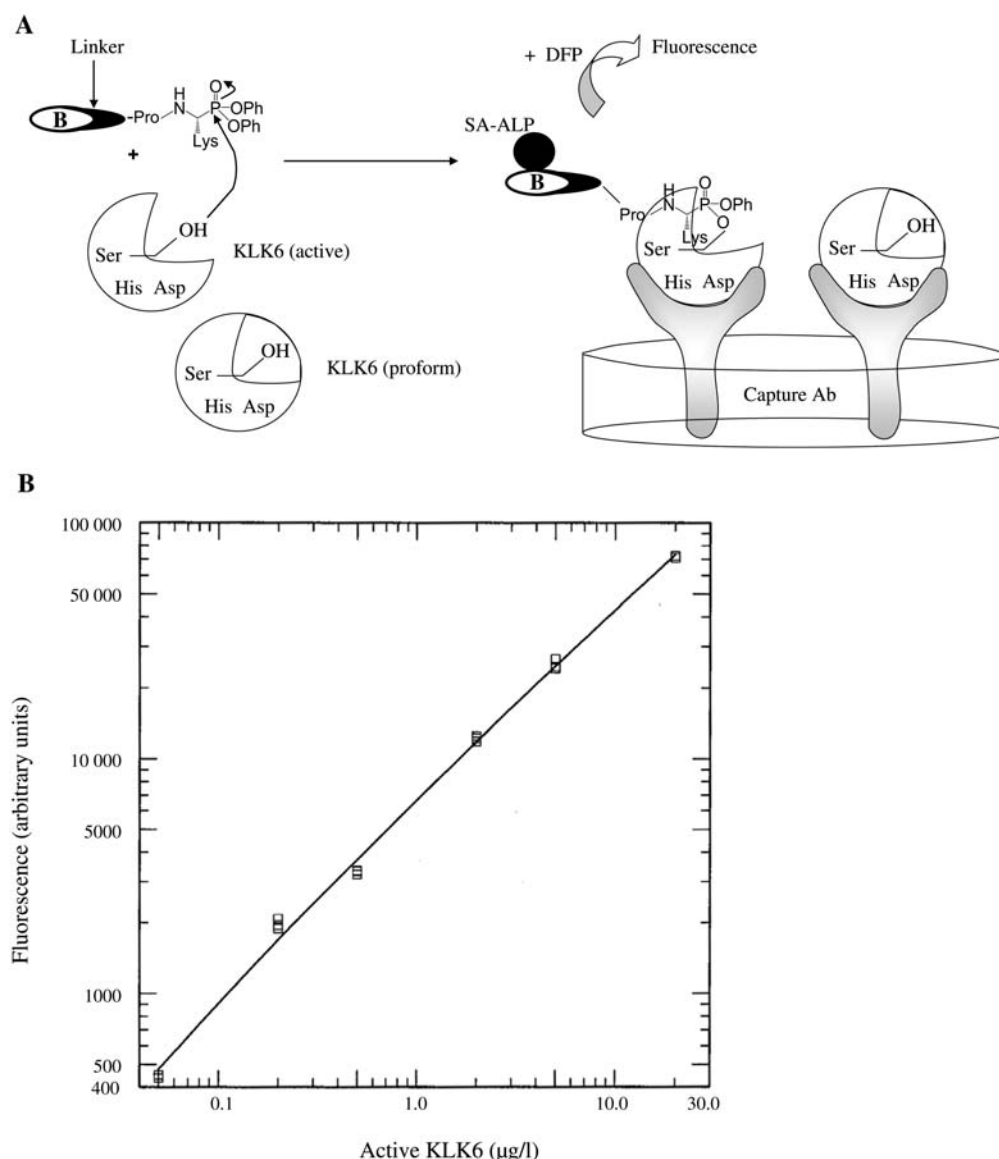


Figure 3 Outline of the procedure for the active KLK6 ELISA assay (A) and calibration curve for detection of active enzyme (B). (A) The scheme illustrates the procedure, as outlined in materials and methods, for covalently labeling active KLK6 with the biotinylated (B, attached to linker: dark oval) proline-lysine-containing ABP (Bio-PK), followed by capture of the biotinylated enzyme with a monoclonal KLK6-targeted antibody (capture Ab). Capture is followed by generation of the fluorescent signal from the bound streptavidin-alkaline phosphatase (SA-ALP) and the DFP substrate. (B) The graph shows a representative standard curve (logarithmic scale on both axes) of the active KLK6 ELISA assay, using enzyme isolated by the new two-step procedure described in the materials and methods section and illustrated in Figure 2.

reactive enzyme (approx. 95–99.9%) appeared not to react with the ABP (biotinylated probe detection relative to total ELISA) and thus the majority of the enzyme was presumably in the inactive state (Figure 4). Ascertaining the molecular forms of the immunoreactive but enzymatically inactive KLK6 remains a topic for further work.

To validate the ability of our assay to recognize the active form of KLK6 in complex mixtures, we supplemented the above-mentioned biological samples with 7.7 μg/l (257 pM) recombinant active KLK6. When added first to a clinical sample prior to biotin labeling, the reactivity of the KLK6 with the ABP was significantly diminished. According to the calibration curve, recovery of the active enzyme from supplemented samples ranged from ≥100% of supplemented concentrations (amount of

enzyme in KLK6-supplemented samples relative to an equal amount of supplemented KLK6 in a sample of 6% BSA buffer) to levels that were below the detection limit of our assay (Figure 4, right Y-axis). In general, the recovery observed for added active enzyme from CSF and ascites samples was higher than for cancer cell supernatants, which yielded a recovery of <50%. We interpret these data as an indication that samples, like those obtained from cancer cell supernatants, contain sufficient inhibitors to bind and inhibit both endogenous active enzyme and exogenous added enzyme. Furthermore, the higher than expected recovery for KLK6-supplemented samples with low endogenous levels of active KLK6 may be the result of further proteinase activation originating from the reservoir of endogenous KLK6 zymogen.

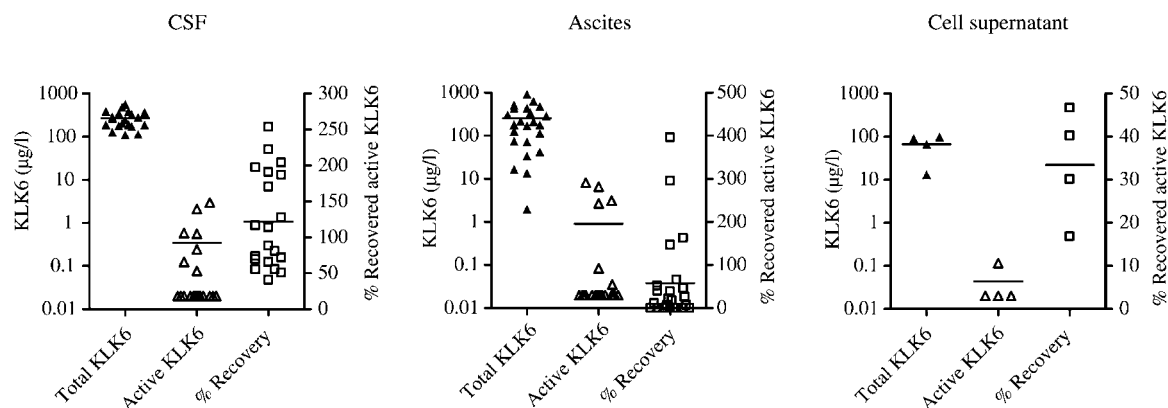


Figure 4 Levels of KLK6 in biological fluids and cultured cell-derived supernatants, measured as total immunoreactive KLK6 (total KLK6) and as active enzyme (active KLK6).

The graphs show values (each sample shown by an individual symbol) measured for samples of cerebrospinal fluid (CSF), ovarian cancer-associated ascites (Ascites) and cell culture supernatants obtained from breast, ovarian and prostate cancer-derived cell lines (Cell supernatant). The horizontal bars in each panel show the average values ($\mu\text{g/l}$ immunoreactive or enzymatically active KLK6: $0.03 \mu\text{g/l} = 1 \text{ pM}$, molecular mass = 30 kDa) derived from measurements of all samples in each group. Also shown on the right of each panel is the recovery of active KLK6 (% recovery of total active KLK6 in supplemented samples relative to an equal amount of supplemented KLK6 in a sample of 6% BSA buffer) for samples to which a known amount of active enzyme was added prior to ABRA-ELISA assay. Note that a log scale is used for concentration on the left-hand Y-axis.

Discussion

The main outcome of our study is that it is now possible to use combined ABP labeling with ELISA-based detection of total KLK to monitor the proportion of enzymatically active enzyme relative to total immunoreactive KLK present in samples derived from clinical or other sources. The general ABP-immunological detection approach (e.g., ABP labeling coupled to Western blot detection) facilitated the development of an efficient method for the purification of fully active KLK6 from a cell expression system that secretes significant amounts of zymogen amongst a variety of other proteins in the serum-free supernatant. The two-step procedure, involving a thermolysin-catalyzed activation of KLK6 as monitored by the ABP reaction, takes advantage of the chromatographic shift in elution of the active enzyme relative to the KLK6 zymogen. The active enzyme generated by thermolysin cleavage elutes at a higher volume relative to that of the zymogen. This shift to higher elution volume of the activated enzyme (e.g., fractions eluting between 65 and 75 min, Figure 2A, lower chromatogram) relative to the zymogen-containing fractions recovered in the first step frees the thermolysin-generated product from contaminating proteins that elute at the position of the zymogen in the first step (e.g., fractions eluting between 40 and 65 min, Figure 2A, upper chromatogram). This straightforward two-step procedure provided the key active-enzyme preparation that was required to validate our quantitative ratiometric assay.

In preliminary work, we established that reaction of the ABP with the enzyme was essentially complete over a timeframe of approximately 4 h (data not shown) and thus routine labeling in the presence of a molar excess of ABP/enzyme was allowed to proceed for twice this time prior to the antibody capture step. The ELISA was able to validate the recovery of enzyme both with and without the labeling procedure, confirming that the labeling step *per se* did not affect the ability of the capture

antibody to recover KLK6 protein from the samples. We are thus confident that the total capture of immunoreactive KLK6 from unknown samples included the same high proportion of biotinylated enzyme that had reacted with the ABP. We did not observe significant degradation of KLK6 protein during the time course of the ABP labeling and ELISA assay (Figure 1, middle gel panel and data not shown).

Our ratiometric approach revealed that the majority of KLK6 quantified by ELISA is enzymatically inactive in CSF, ovarian cancer ascites fluid and tumor-derived cell culture supernatants. This result is in agreement with previous studies reporting that KLK6 exists mainly as a zymogen in biological fluids (Okui et al., 2001; Hutchinson et al., 2003; Luo et al., 2006). Nevertheless, in all three types of biological samples we were able to label a small but significant amount of enzyme with the ABP. This relatively low concentration of active enzyme may not have been detected with methods that we used previously (Figure 4). The proportion of active enzyme relative to total immunoreactive protein was low in the samples we surveyed (approx. 0.1–5%). However, even though the absolute concentrations of active enzyme in our clinical and cell-derived samples were low (~ 0.04 –8 U/l of trypsin-like activity; see Materials and methods), the total amount of enzyme in the samples, if generated in a restricted environment, would in principle be sufficient to cause cell and tissue signals either by cleaving and activating PARs (Oikonomopoulou et al., 2006a,b) or via other mechanisms. For example, the active enzyme produced by tissues at a localized site could, as well as regulating PARs, be present in concentrations able to cleave extracellular matrix molecules or myelin-related proteins, all of which have been established as targets of KLK6 *in vitro* and could, in principle, contribute to disease pathogenesis (Blaber et al., 2002; Scarisbrick et al., 2002; Magklara et al., 2003; Borogio and Diamandis, 2004; Oikonomopoulou et al., 2006a,b).

To the best of our knowledge, this is the first study that combines the high throughput and high sensitivity of an ELISA with the advantages of an ABP analysis.

The low recovery of active enzyme able to react with the ABP reagent in clinical samples supplemented with pure active KLK6 merits comment. We believe that the added enzyme activity is rapidly quenched by the presence of serine proteinase inhibitors (serpins; Law et al., 2006) known to be present in such samples. For example, antitrypsin and inter- α -trypsin inhibitor are elevated in cancer (Kuramitsu and Nakamura, 2006), in which KLK6 is also present (Yousef et al., 2004; Nagahara et al., 2005). In addition, antitrypsin and α 2-macroglobulin (a general proteinase inhibitor) have been identified in CSF, along with KLK6 (Dumont et al., 2004; Yuan and Desiderio, 2005). *In vitro* studies have also shown that KLK6 can bind to α 2-antiplasmin and antithrombin III (Magklara et al., 2003) and a KLK6- α 1-antichymotrypsin complex has been described in ascites fluid (Hutchinson et al., 2003).

In summary, the new ratiometric assay approach described here can identify both active and inactive forms of KLKs in clinical samples and may therefore serve as a novel diagnostic tool to identify the active form of KLKs in pathophysiological settings. We hypothesize that measurement of the proportion of active KLKs in clinical samples will provide information that is complementary to the current KLK ELISA measurements now being used as biomarkers for disease diagnosis and prognosis.

Materials and methods

Reagents

Diflunisal phosphate (DFP) was synthesized in our laboratory and a stock solution of 0.01 M in 0.1 M NaOH was prepared. Thermolysin was obtained from Sigma (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada; 44 U/mg solid or 77 U/mg protein; 1 U hydrolyzes casein to produce color equivalent to 1 μ mol/min at pH 7.5 and 37°C). Porcine trypsin was also purchased from Sigma (catalog no. T-7418; 14 900 U/mg). The activity-based probe used to detect serine proteinase activity was provided by Dr. Amos Baruch (KAI Pharmaceuticals, South San Francisco, CA, USA). The probe was designed to specifically target the active site of serine proteinases with trypsin-like activity (e.g., biotin-linker-Pro-Lys-diphenylphosphonate, Bio-PK) (Pan et al., 2006). KLK6 enzyme activity was measured as previously described (Magklara et al., 2003) using the fluorogenic synthetic substrate VPR-AMC (Bachem Bioscience, Inc., King of Prussia, PA, USA).

Biological samples

All clinical specimens were collected after Ethics Approval from the Mount Sinai Hospital Institutional Review Board. Ascites samples were collected from ovarian cancer patients at Princess Margaret Hospital, Toronto, Canada. Residual CSF samples in excess of the amounts required for routine testing were obtained at Mount Sinai Hospital, Toronto, Canada. Two ovarian cancer cell lines, HTB-75 (Caov-3) and HTB-161 (OVCAR-3), and one breast cancer cell line, HTB-132 (MDA-MB-468), were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The androgen receptor-transfected PC-3(AR)₆ cell

line was a generous gift from Dr. Theodore Brown (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, and the University of Toronto, Toronto, ON, Canada) (Heisler et al., 1997). All cell lines were cultured in RPMI 1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with fetal bovine serum (10%) in 75-cm² flasks. Cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere until cell monolayers reached confluence, after which supernatants were collected. All samples were stored at -20°C until use.

Production, purification and activation of recombinant KLK6

Human embryonic kidney (HEK-293) cells transfected with the zymogen form of KLK6 were grown in serum-free medium as previously described (Little et al., 1997; Magklara et al., 2003). Pure KLK6 zymogen (inactive proform) was purified as described by Magklara et al. (2003). In brief, the tissue culture supernatant was collected and concentrated 10-fold using a Centricon ultrafiltration device (Millipore, Waltham, MA, USA), with a 10-kDa cutoff nitrocellulose membrane (Millipore). The concentrated supernatant was then diluted two-fold in running buffer (50 mM sodium acetate, pH 5.3) and the pH was adjusted to 5.3 before analyzing the sample by ion-exchange liquid chromatography (ÅKTA™ FPLC; GE Healthcare Bio-Sciences, Uppsala, Sweden) using either protocol A for production of KLK6 zymogen and subsequent conversion to its active form or protocol B for direct production of active KLK6 from tissue culture supernatants.

Protocol A: production of KLK6 zymogen To purify the KLK6 zymogen, we loaded the concentrated supernatant onto an ion exchange column (5-ml HiTrap CM FF column, GE Healthcare Bio-Sciences) as previously described (Magklara et al., 2003). FPLC fractions were analyzed by ELISA and those containing KLK6 were pooled, supplemented with 1% trifluoroacetic acid, loaded onto a 1-ml C4 reverse-phase column (VYDAC® 214TP; Grace, Deerfield, IL, USA) and separated by reverse-phase HPLC (Agilent 1100, Agilent Technologies Inc., Santa Clara, CA, USA). Elution was performed using a linear gradient of 0–100% acetonitrile in 0.1% trifluoroacetic acid in deionized H₂O.

The KLK6 zymogen was activated by incubation for up to 2 h with thermolysin at a 1:115 molar ratio (thermolysin/KLK6) in 50 mM Tris, 10 mM CaCl₂, 150 mM NaCl, pH 7.5, at room temperature. The reaction was terminated by addition of 50 μ M EDTA, followed by rapid freezing with liquid nitrogen. The conversion from proform to active enzyme was monitored by incubation with 5 nM Bio-PK for 2 h at room temperature and analysis by SDS-PAGE under reducing conditions (NuPage 4–12% Bis-Tris gels; Invitrogen, Burlington, ON, Canada). Biotinylated proteins were transferred to a Hybond-C Extra nitrocellulose membrane (Amersham Biosciences, Pittsburgh, PA, USA) and blocked with 5% casein and 0.1% Tween-20 in Tris-buffered saline (TBST) for 2 h at room temperature. Membranes were then washed briefly with TBST, treated for 1 h with 50 ng/ml SA-ALP diluted in 1% casein-TBST, washed and visualized with Immulite chemiluminescent substrate treatment for 10 min (Diagnostic Products Corp., Los Angeles, CA, USA). This Western blotting detection of ABP-proteinase complexes has been described previously (Hansen et al., 2005).

Protocol B: direct isolation of active KLK6 To purify active KLK6 directly from concentrated cell culture supernatant, we used the ion-exchange liquid chromatography protocol described above, slightly modified to include a new two-step chromatographic isolation procedure with a sodium chloride gradient. The concentrated supernatant was diluted two-fold in running buffer as above and loaded onto the FPLC column (5-

ml HiTrap SP HP column, GE Healthcare Bio-Sciences) at a flow rate of 0.8 ml/min. Fractions of 4 ml were eluted at 3 ml/min using a three-step gradient of 10% (15 column volumes), 20% (15 column volumes) and 30% (10 column volumes) of elution buffer (1 M NaCl in 50 mM sodium acetate, pH 5.3). Fractions were analyzed by ELISA and SDS-PAGE under reducing conditions. Protein in the SDS-PAGE gels was monitored (data not shown) by Western blotting (KLK6 antibody), Coomassie staining (SimplyBlue Safestain; Sigma) and biotin-ABP labeling as described in Protocol A. The enzyme activity of fractions was also assessed using the fluorogenic synthetic substrate VPR-AMC as previously described (Magklara et al., 2003). Enzyme activity reactions were performed in 50 mM Tris, 0.1 mM EDTA, 0.1 M NaCl, 0.01% Tween-20, pH 7.6, at 37°C. Fluorescence was monitored for 20 min on a Wallac Victor fluorometer (Perkin-Elmer, Wellesley, MA, USA) set at 355 nm for excitation and 460 nm for emission.

Fractions containing the KLK6 zymogen (Western blot and enzyme activity analysis) were pooled and incubated with 5.8 U of thermolysin (75 µg) at a 1:115 thermolysin/KLK6 molar ratio. The reaction was allowed to proceed for 2 h at room temperature and pH 7.5. Following incubation, the sample was diluted two-fold in running buffer, the pH was adjusted to 5.3 and the sample was loaded onto a 5-ml HiTrap SP HP FPLC column. The same salt gradient was run and FPLC fractions were collected and analyzed as described above. We also incubated the fractions with 5 nM Bio-PK, as in protocol A, to monitor serine proteinase activity (Hansen et al., 2005). The fractions containing active KLK6 were combined and concentrated 10-fold in 50 mM sodium acetate, pH 5.3, using 10-kDa cutoff Amicon concentration tubes (Millipore). Protein purity was accessed by SDS-PAGE and detailed enzyme kinetic analysis was performed using VPR-AMC (Magklara et al., 2003).

Combined ABP-ELISA assay of KLK6-containing samples

Each sample was assayed independently (a) for total immuno-reactive KLK6 measured using an in-house ELISA and (b) for total ABP-reactive biotinylated KLK6 measured using a combined antibody capture/streptavidin-based biotin microtiter plate assay. A standard curve for KLK6 was prepared for these assays using the KLK6 enzyme prepared by our new two-step purification procedure.

KLK6 sandwich-type ELISA The sandwich-type ELISA for measuring total KLK6 used an antibody developed in our laboratory, as described elsewhere in detail for its KLK6 selectivity and lack of cross-reactivity with other kallikrein-related peptidases (Diamandis et al., 2000c; Luo et al., 2006). In the present study, two in-house monoclonal antibodies against KLK6 were utilized (Luo et al., 2006). A time-resolved fluorometer, the CyberFluor 615 Immunoanalyzer (MDS Nordion, Kanata, ON, Canada), was used to measure Tb³⁺ fluorescence in white 96-well microtiter plates. The assay principle has been described in detail elsewhere (Christopoulos and Diamandis, 1992; Ferguson et al., 1996).

KLK6 ABRA-ELISA Several ABPs for trypsin-like serine proteinases available in our laboratory were screened to select the probe with the highest sensitivity for KLK6. More specifically, we utilized four biotinylated diphenylphosphonate probes containing Pro-Lys, Lys-Lys, Asp-Lys and succinyl-Lys as the P₂ and P₁ residues, respectively. The first three probes exhibited similar sensitivity and we therefore decided to continue the study using the Pro-Lys form (biotin-linker-Pro-Lys-diphenylphosphonate or Bio-PK) only.

KLK6 calibrators were prepared by diluting recombinant active KLK6 protein produced as described above [activity equal to 3500 nmol of VPR-AMC/(min×mg of KLK6), which is equivalent to 1 unit (U) of trypsin-like activity per µg of KLK6 when compared to trypsin against VR-AMC; Oikonomopoulou et al., 2006a] in bovine serum albumin (BSA) buffer (6% BSA, 50 mM Tris, 0.05% sodium azide, pH 7.8). Where appropriate, sample dilutions were also performed in 6% BSA buffer. Coating antibody solution (100 µl of 50 mM Tris buffer, 0.05% sodium azide, pH 7.8, containing 500 ng of antibody/well) was added to each well and incubated for 8 h at room temperature as previously described (Diamandis et al., 2000c). Calibrators or samples of unknown active KLK6 content were diluted two-fold with 5 µM ABP in KLK6 activity buffer (50 mM Tris, 0.1 mM EDTA, 0.1 M NaCl, 0.01% Tween-20, pH 7.6) and incubated for 8 h at room temperature prior to addition to the antibody capture plate. Plates coated with a KLK6-selective monoclonal antibody (Luo et al., 2006) were first washed twice with washing buffer (5 mM Tris, 150 mM NaCl, 0.05% Tween-20, pH 7.8) and the ABP-reacted standards or unknown samples were added and incubated overnight at room temperature. Subsequently, 5 ng/well of SA-ALP solution (Jackson ImmunoResearch, West Grove, PA, USA) in BSA buffer was added to each well and incubated for 15 min at room temperature. The plate was washed six times with washing buffer and the substrate (100 µl of 0.1 M Tris buffer, pH 9.1 containing 1 mM DFP, 0.1 M NaCl and 1 mM MgCl₂) was added to each well and incubated for 10 min at room temperature. Finally, developing solution (100 µl of 1 M Tris, 0.4 M NaOH, 2 mM TbCl₃, 3 mM EDTA) was added to each well and incubated for 1 min at room temperature. The fluorescence was measured on a CyberFluor 615 Immunoanalyzer. Calibration and data reduction were performed automatically.

The detection limit of this immunoassay and the specificity against KLK6 were determined using different ratios of active to inactive protein of all forms (proform and/or degradation products). We also evaluated the sensitivity and specificity of the coating monoclonal antibody used to capture KLK6 in the ABP ELISA assay after reacting with the ABP.

The ability to measure the concentration of active (ABP-reactive) relative to total (regular ELISA) KLK6 permitted the calculation of the percentage of the active and ABP-reactive form of KLK6 (KLK6 ABRA-ELISA).

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