Isolation and characterization of Stromelysin-3(MMP-11) like protein from a case of mammary carcinoma in a dog

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ABSTRACT

Stromelysin-3(MMP-11) is a matrix-metalloproteinase known to be over expressed in invasive tumors of human origin and suppose to have a potential role in metastasis. Dog mammary tumors are usually very invasive. Thus to characterize dog stromelysin-3, mammary tumor tissue (papillary adenocarcinoma) was used to isolate the protein by using affinity chromatography tools including Cibacron-Blue 3GA-CL Agarose, gelatin Sepharose, and heparin Sepharose columns. Western blotting with antihuman stromelysin-3 antibody was done for identification. The result showed a heavier (120kDa) protein than the expected size of stromelysin-3. Western blot analysis of the crude sample did not show any other reactive bands. Under reducing condition, the protein size remained intact. The stromelysin-3 like protein showed mild caseinolytic activity, which increased by activators like APMA and trypsin. Whereas, its activity decreased when inhibitors like EDTA, 1, 10-Phenanthroline and TIMP-1 were used suggesting the requirement of calcium and zinc for enzyme activity just like any other metalloproteinase.

Key words: Metastasis, MMP-11, Papillary adenocarcinoma, Stromelysin-3, TIMP-1.

Cancer is one of the dreaded disease conditions of living organisms. With reference to the incidence, dog is the most frequently affected domestic species, with skin and mammary tumors having highest incidence (Rungsipipat et al., 2003). Malignant mammary tumors usually metastatise causing death of the patient. Dissolution of extracellular matrix (ECM) is an essential step for cancer cell metastasis. The proteinases primarily involved in tumor cell metastasis are the matrix metalloproteinases (MMPs) and plasminogen activators (Garbett et al., 1999). The MMPs, also called as matrixins, are a family of structurally and functionally related, zinc dependent endoproteinases that are involved in the dissolution of the ECM. MMPs are divided into four main subgroups: the interstitial collagenases, gelatinases, stromelysins and membrane MMPs (Chambers and Matrisian, 1997; Duffy and McCarthy, 1998). The stromelysins, consist of stromelysin-1 (MMP-3), stromelysin-2 (MMP-10), stromelysin-3 (MMP-11) and matrilysin (MMP-7).

Stromelysin-3, a member of this family, was first identified by subtractive hybridization using breast carcinoma and fibroadenoma cDNA libraries and was found to be specifically over expressed in breast carcinoma (Basset et al., 1990). In the case of breast carcinomas, elevated stromelysin-3 mRNA levels in the primary tumor are highly predictive for the presence of distant metastases (Engel et al., 1994). Stromelysin-3 is expressed in most invasive carcinomas (Rouyer et al., 1994) and high levels of expression indicate a poor prognosis. On the other hand, Andarawewa et al. (2003) reported that ras+/+: ST3-/- (deficient) mice developed more metastases, thus the dual role of stromelysin-3 in carcinogenesis and metastasis arouse a lot of interest to find out its exact role and target substrates. Although the enzyme has been so named because of its homology to members of a family of matrix-degrading MMPs; Stromelysin-3 has certain exceptions. It has not yet been found to degrade any matrix protein and unlike other MMPs, the stromelysin-3 proenzyme is released as a mature enzyme (Pei and Weiss, 1995). Realizing the highest incidence of dog mammary tumor and important role of stromelysin-3 in carcinogenesis and metastasis, the current study was undertaken to identify and characterize the dog stromelysin-3.

Dog mammary tumor tissues: Canine mammary tissue samples were collected at the time of surgery carried out at the Laparoscopy Section, Veterinary Polyclinic, IVRI, Izatnagar, India. The tumor tissue were collected under aseptic condition and brought to laboratory on ice. Part of the tissue was sent for histopathological examination and rests were stored at -20°C until further processing.

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Purification of stromelysin-3: Stromelysin-3 was purified using a combination of chromatographic methods as described by Pei et al. (1994) with some modifications. All the purification procedures were carried out at 4°C with 50mM Tris-HCl, pH 7.5 buffer containing 5mM CaCl₂, 0.05% Brij-35 and 0.02% Na₂SO₄. The tumor tissue was subjected to mechanical homogenization in a ratio of 1:3 to ice cold buffer. The homogenate was subjected to centrifugation at 12,500 rpm for 15 minutes at 4°C. The supernatant was syringe filtered and used for protein purification. The protein concentration at each step was monitored by Bradford assay or UV method. After passing the supernatant in Cibacron-Blue 3GA-CL Agarose column, the bound protein was eluted in equilibration buffer containing 1M NaCl in 2ml fractions. The more protein containing eluted fractions were then dialyzed with equilibration buffer and applied to Gelatin Sepharose column. Flow through was collected, dialysed and applied to the Heparin Sepharose column. After sample loading and washing with the equilibration buffer, the bound proteins are eluted through a gradient of 0-1M NaCl.

Western blotting: The nitrocellulose membrane after electrotransfer (Bangalore Genei) was blocked in 5% skimmed milk solution. Following 3-4 washings with PBS-Tween20, primary antibody (developed against MMP-11 of human in rabbit, Prolabs) was added at a dilution of 1:500 and incubated at 37°C for 1½ hr with gentle agitation. After further washings, secondary antibody (goat anti rabbit immunoglobulin) at the same dilution was added and incubated at 37°C for 1hr with gentle agitation. Rabbit anti-goat ALP conjugate at the dilution of 1:2000 was then added to the nitrocellulose membrane, after washing it and has incubated at 37°C for 1 hr with gentle agitation. Final washings were done and the substrate (BCIP/NBT, Amresco.) was applied to the membrane in dark and the color reaction developed within 1 hour.

SDS-PAGE gelatin zymography: Gelatin degrading enzymes present in the fractions degrade the gelatin matrix, leaving a clear band after staining the gel for protein (Huessen and Dowdle, 1980). The resolving gel (8%) was copolymerized with 0.3% gelatin solution (0.15% final concentration). Samples are loaded onto the gel mixed with sample buffer without boiling. Then, renaturation was carried out in renaturation solution (2.5% TritonX-100) for 3 hours on a mechanical shaker with mild agitation. The gel has incubated in developing buffer (10mM CaCl₂, 0.15M NaCl and 50mM Tris, pH 7.5) for 18 hours at 37°C and then stained with 0.25% coomassie blue for 2 hours, followed by destaining. For calibration gelatin zymograms with human gelatinase standards, the method described by Makowski and Ramsby (1996) was followed.

SDS-PAGE casein zymography: The resolving gel (11%) was copolymerized with casein (2mg/ml final concentration). Rest of the procedures was same as gelatin zymography.

Activation by 4-amino-phenyl mercuric acetate: 20µg of the partially purified protein was taken in two clean eppendorf tubes. To one of the tubes, 50µl of 1mM APMA prepared in buffer A (50mM Tris-HCl, 5 mM CaCl₂, 200mM NaCl, and 0.1%Na₂SO₄ (pH 7.5)) was added. The other one was used as control and both the tubes were incubated at 37°C for 16 hrs. Then the tubes were removed and aliquots from each tube subjected to casein zymography.

Trypsin activation: To 20µg of the partially purified protein in a clean eppendorf tube, trypsin was added at the concentration of 1µg/ml and incubated at 25°C for 15 min. The reaction was stopped by adding 10-fold excess of soybean Trypsin inhibitor. The aliquot was subjected to casein zymography.

Effect of inhibitors on the activity of the purified protein: 20µg of partially purified protein were taken in four eppendorf tubes. 20 il of each EDTA (10 mM), 1, 10-Phenanthroline (1mM) (prepared in buffer A) and TIMP-1 (0.1mg/ml) were added to individual tubes and one tube without any inhibitor was kept as control. The tubes are incubated at 37°C for a period of 16 hours. Aliquots from each tube were subjected to gelatin and casein zymography.

Metastasis is the main concern in the prognosis of cancer. Of particular interest here is the stromelysin-3 in dog. Because of its increased expression in tumors, in this study purification and characterization of stromelysins-3 was attempted from dog mammary tumor tissues (papillary adenocarcinoma confirmed after histopathological examination). The total protein concentration was found to be 1.29mg/ml in the crude homogenate. The activated dye of the Cibacron Blue 3GA-CL Agarose column binds MMPs along with some other proteins. The large amount of unwanted proteins passed out in the flow through. The Cibacron Blue 3GA-CL Agarose column elution fractions showing high protein concentration (1.55 mg/ml, 1.67mg/ml, 0.4mg/ml) were used further. The presence of the endogenous gelatinases could mask the presence of stromelysin-3 in the zymographic analysis (Pei et al., 1994), so sample was depleted of gelatinases by gelatin affinity chromatography. Gelatin Sepharose affinity column is a valuable tool for the isolation of gelatinases with very high binding potential with binding affinity of 10mg/ml. The flow through collected is re-passed in the column to be assured of removal of all traces of gelatin binding proteases. The protein concentration was found to be 0.46mg/ml in the flow-through. MMPs including stromelysin-3 having the haemopexin like domain bind to
heparin. The maximum 120kDa protein with concentration 0.6mg/ml was eluted at 0.1M-0.2M NaCl in Heparin Sepharose column Chromatography.

Heparin Sepharose eluents showed a mixture of proteins on SDS-PAGE (Fig.1) and on western blot analysis (Fig.4), a 120 kDa band was detected. Pei et al. (1994) reported human stromelysins-3 of size ranging from 20 to 65kDa with a major 45 kDa species and an intact C terminal domain, identified as a true endopeptidase. Mari et al. (1997) reported a 35kDa form of stromelysins-3 as the most abundant stromelysins-3 protein in tumor/stroma co-cultures. Western blot analysis of crude tumor tissue sample (Fig.3) also showed no other prominent reactive band except the 120kDa protein ruled out the possibility of its cross reactivity with any other protein. On SDS-PAGE under reducing conditions followed by western blot analysis, no change in the size of 120kDa protein band was observed which ruled out the possibility of some polymeric forms. Murphy et al. (1993) reported the cross reactivity of mouse stromelysin-3 with human stromelysin-3. Stromelysin-3 was known to be conserved in several phyla suggesting significant cross reactivity between human and canine stromelysin-3. Esther et al. (2001) quoted gelatin as a substrate for stromelysins-3. Ogiwara et al. (2002) reported gelatin-degrading activity of fish stromelysins-3. But there was no appreciable gelatinase activity observed on gelatin zymography of gelatin Sepharose flow-through (Fig.2). On the other hand, low caseinolytic activity of the purified 120kDa protein was observed by casein zymography. Truncated segments of mouse stromelysin3 were reported to have caseinolytic activity and activation of stromelysin-3 of mouse by APMA and trypsin has been reported earlier (Murphy et al., 1993). On activation with APMA and trypsin, there is increase in caseinolytic activity of the 120kDa protein compared to untreated enzyme with appearance of low molecular weight active forms (Fig.5). 120kDa protein underwent partial autocatalytic processing following incubation with APMA (Birkedel-Hansen et al., 1993) and trypsin. Trypsin activation shows more increased activity than APMA activation. The caseinolytic activity of the 120kDa protein were completely inhibited by inhibitors like EDTA (10 mM), 1,10-Phenanthroline (1mM) and TIMP-1 (0.1mg/ml). Mouse stromelysin3 proteolytic activity on casein zymography was completely inhibited by EDTA, 1, 10-Phenantherline and TIMP-1 (Murphy et al., 1993). The inhibition of caseinolytic...
FIG 3: Western blot analysis of crude tumor tissue sample using antihuman stromelysin-3 antibody
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activity by EDTA, Ca chelator (Lana et al., 2000) and by 1, 10-Phenanthroline, a zinc chelator (Makowski and Tamsby, 1996), suggested that the enzyme was a calcium and zinc dependent MMP.

REFERENCES


