Cibtech Journal of Bio-Protocols ISSN: 2319–3840 (Online)
An Online International Journal Available at http://www.cibtech.org/cjbp.htm
2013 Vol. 2 (1) January-April, pp.8-21/Krupakaran
Research Article

ISOLATION AND CHARACTERIZATION OF 220 KDA MMP -9 FROM CANINE MAMMARY TUMOR

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ABSTRACT
The pattern and level of expression of MMP-9 in sera and tumor tissue of canine mammary tumours were studied. 220 kDa, 135 kDa and 92 kDa are the three major forms of MMP-9 predominantly present in sera, neutrophils and tumor tissue of dogs. Isolation and purification of MMP-9 was carried out from canine tumor tissue homogenate and canine neutrophils. In gelatin sepharose chromatography, MMP-9 and MMP-2 were recovered to 9.19% from neutrophils and 25.56% from tumor tissue homogenate respectively. In con-A sepharose chromatography, MMP-9 was separated from MMP-2 and the yield was 6.50% and 16.21% for neutrophils and tissue homogenate respectively. In heparin sepharose chromatography, 220 kDa form MMP-9 was recovered in 0.1 M NaCl elution from 135 and 92 kDa forms and the yield was 3.7% and 6.21% for neutrophils and tissue homogenate respectively. Biochemically, the enzyme was found to be completely inhibited by EDTA and 1, 10-phenathroline and it required optimum pH of 7.2 -7.7 and optimum temperature of $37^{O}C$-$42^{O}C$ and divalent cation Ca^{2+} for its activity. Canine MMP-9 cross-reacted with anti-human MMP-9 antibodies. The antibody raised against 220 kDa homodimer form of MMP-9 identified all the three forms of MMP-9 (92,135 and 220 kDa). Clinical studies identified that the level of expression of MMP-9 was 2.137 and 6.48 fold higher in sera and tumor tissue of diseased dogs.

Key Words: Matrix Metalloproteinase-9, Canine Mammary Tumor, Gelatin Zymography, Affinity Chromatography, Western Blotting

INTRODUCTION
Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases that play important roles in morphogenesis, tissue remodelling, reproduction and control of cell behaviour under physiological conditions (Birkadel – Hansen *et al*., 1993). In excess, they may participate in accelerated pathological destruction of extracellular matrices associated with various connective tissue diseases and cancer cell invasion and metastasis (Declerck, 2000 and Steen *et al*., 2002). The metalloendopeptidases family consists of mainly four groups, as, collagenases, gelatinases, stromelysins and membrane type MMPs (Nagase and Woessner, 1999). Among these four groups, the two gelatinase enzymes, MMP-2 and MMP-9 have been associated with malignant tumour progression and metastasis. Higher expression of MMP-2 and MMP-9 expression was reported in malignant mammary tumours of dogs (Lana *et al.,* 2000). Further reports suggest that the MMP-2 may activate the MMP-9 through a membrane type MMP (Curran and Murray, 1999 and Dzwonek *et al.,* 2004). Tumours of mammary gland are the most common tumours of female dogs representing approximately 30-50 % of all tumours in bitch (O'Keefe, 1995). There was ratio of 1:19.23 dogs to human and mean number of dogs per household was 1:11 (Jayakumar, 1997). MMP-2 was isolated from fibroblasts, chondrocytes and myelomonocytic cell line (Coughlan *et al.,* 1998).

The present paper deals with isolation and characterization of MMP-9 in canine mammary tumour tissue samples.

MATERIALS AND METHODS

A total of 10 fresh canine tissue samples, each weighing about 50 g were collected at the time of surgery carried out in the polyclinic, IVRI, Izatnagar. Tissue samples included tumour and tissues surrounding the tumour. Samples were carried out hygienically in ice and were stored at -70^{0}C, until processed. Part of each tissue sample was fixed in 10% neutral buffered formalin, routinely processed and embedded in paraffin blocks. The sections of the tissue were stained with haematoxylin and eosin (H & E) for the purpose of diagnosis and sent for histopathological examination.

Identification of MMP-9

The method suggested by Lana and co-workers (2000) was followed to identify the presence of MMP-9 activity in the tumour tissue. Samples were homogenized using a mechanical homogenizer in 4 ml of cold 50 mM Tris-buffered saline. Samples were centrifuged for 10 minutes at 1500 x g at 4^{0}C and the supernatants were harvested and preserved at -70^{0}C until further use. Protein estimation was done by the method of Lowry *et al*., (1951).

Gelatin Zymography

The presence of MMP-9 activity in the homogenized tissue sample was confirmed by gelatin zymography (Heussen and Dowdle, 1980). Gelatin (0.15% final concentration) was co-polymerized with non-reducing, denaturing SDS-PAGE (Laemmli, 1970). The resolving gel (8%) was co-polymerized with 0.3% gelatin solution (final concentration of gelatin in gel was 0.15%) and the electrophoretic run was carried out at 100 V until tracking dye reaches the bottom. Then, renaturation was carried out with renaturation solution (2.5% Triton –X 100) for 3 hours on a mechanical shaker with mild agitation. Then, the gel was developed by incubating the gel in developing buffer (10 mM $CaCl_2$, 0.15 M NaCl, 50 mM Tris, 0.02% Brij-35 pH 7.5) for 18 hours at 37^{0}C and the gels were stained with 0.25% Coomassie blue for 2 hours, followed by destaining for 1 hour with destaining solution and then, further destaining was carried out with distilled water, the bands appear clear.

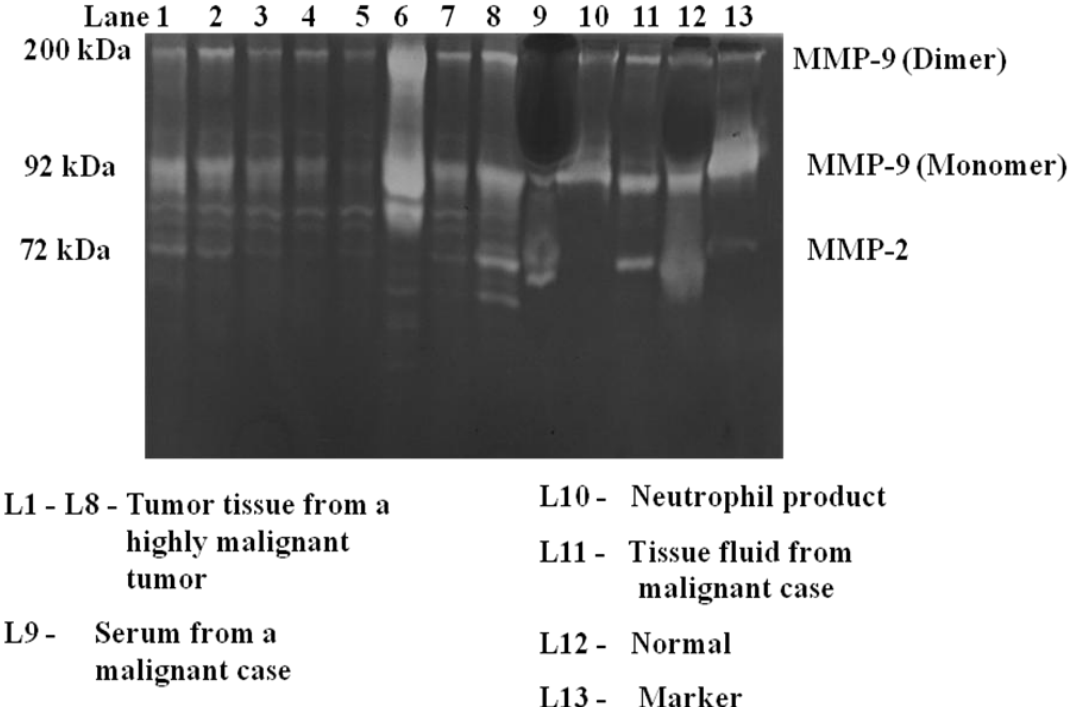


Figure 1: Gelatin zymograms of canine mammary tumor tissues

Calibration of gelatine zymograms were carried out with human capillary blood gelatinase as standards (Makowski and Ramsby, 1996).

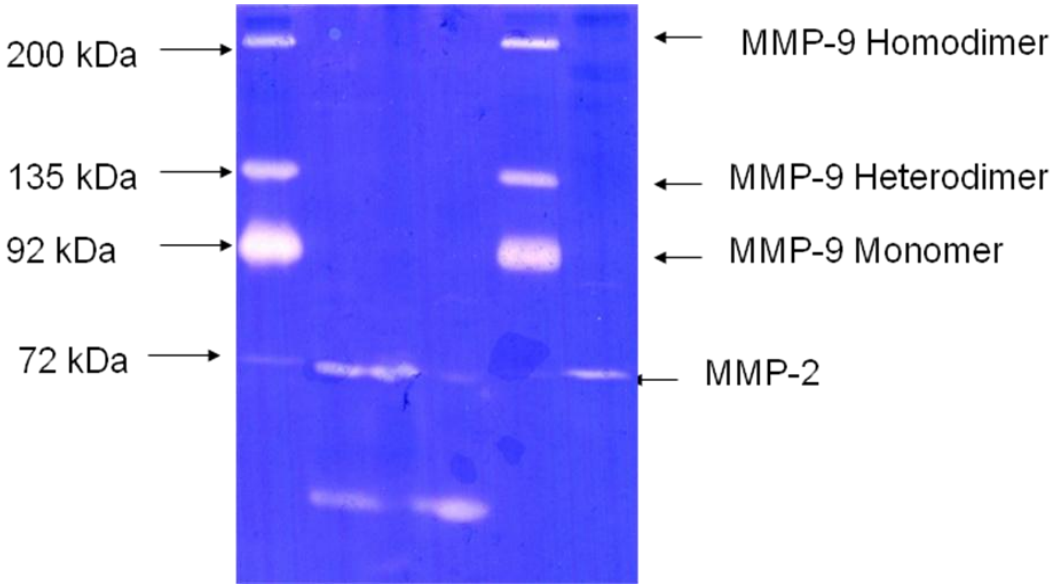


Figure 2: Calibrating gelatin zymograms with human capillary gelatinases

Isolation of MMP-9

220 kDa MMP-9 was purified from tumor tissue homogenate by a 3 step protocol involving a series of chromatography involving gelatin sepharose, con-A sepharose and heparin sepharose. All the purification procedures were carried out at 4^0C with 50 mM Tris-HCl, pH 7.5 buffer containing 10 mM $CaCl_2$, 0.05% Brij-35 and 0.02% NaN_3 unless otherwise stated.

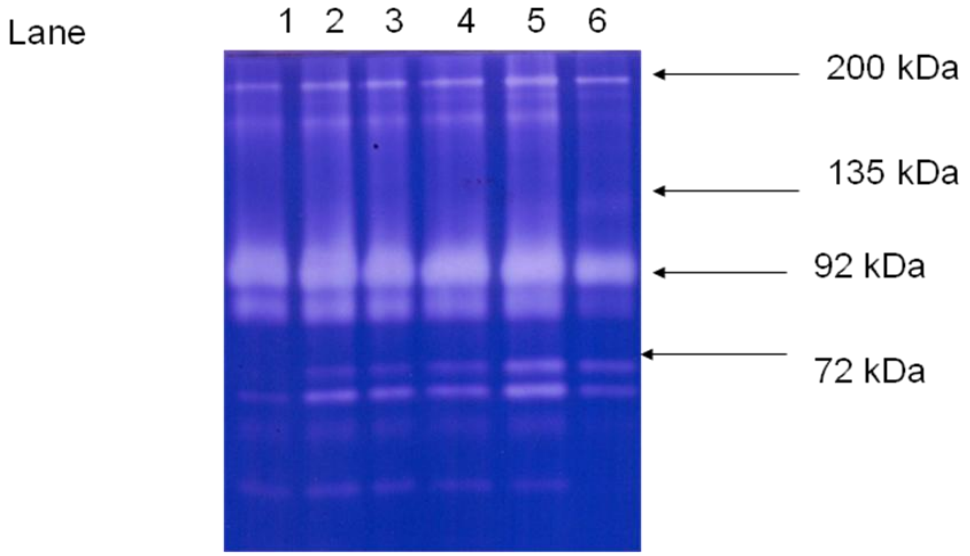
Gelatin Sepharose Chromatography of Mmp-9

The method suggested by Masure and Co-workers (1991) was adopted with some modifications. The column was packed with 4 ml of gelatine sepharose (Sigma) at 4^0C and the column was washed with 50 ml of equilibration buffer (0.05 M Tris-Cl buffer pH 7.6 containing 0.5 M NaCl, 0.005 M CaCl2, 0.05% Brij-35, 0.02% sodium azide and 10 mM EDTA). The weighed tumor tissue wa homogenized in equilibration buffer and the samples were subjected to centrifugation at 12,500 rpm at 4^0C for 15 min. The clear tumor tissue homogenoate was applied to the gelatin sepharose column at a flow rate of 25 ml/hour. Washing of the unbound material was carried out with washing buffer (0.05 M Tris-Cl buffer, pH 7.6 containing 1.0 M NaCl, 0.005 M CaCl2, 0.05% Brij-35, 0.02% sodium azide and 10 mM EDTA). Then, elution of bound MMP was carried out with elution buffer ((0.05 M Tris-Cl buffer containing 0.5 M NaCl, 0.005 M CaCl2, 0.05% Brij-35, 0.02% sodium azide, 10 mM EDTA and 10% DMSO). The absorbance of the eluents was read at 260 and 280 nm and aliquots from the eluents were subjected to both SDS-PAGE (reducing and non-reducing conditions) and gelatin zymography. Then, the eluents were pooled and subjected to concanavalin-A sepharose chromatography.

Concanavalin –A sepharose chromatography of MMP-9

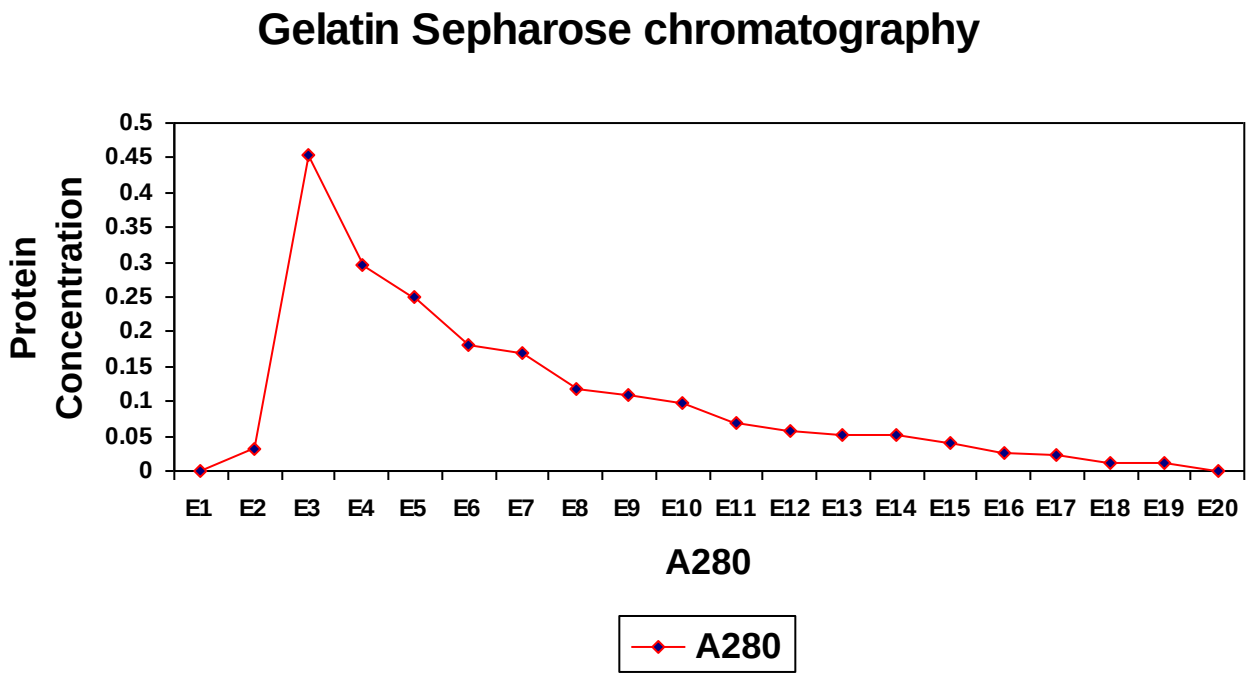
The method suggested by Mandal *et al.,* (2003) was followed with some modifications. The eluents, showing gelatinise activity were pooled and subjected to dialysis against 200 ml of equilibration buffer of con-A sepharose chromatography (0.05 M Tris-Cl buffer pH 7.6 containing 0.15 M NaCl, 0.010 M CaCl2, 0.05% Brij-35 and 0.02% sodium azide) with mild and constant agitation on a magnetic stirrer at 4^0C. The dialysed sample was applied to a 4 ml Con-A sepharose column, which was already1 packed and equilibrated with equilibration buffer. Elution of bound MMP-9 was carried out with a elution buffer (0.05 M Tris-Cl buffer, pH 7.6 containing 0.15 M NaCl, 0.010 M CaCl2, 0.05% Brij-35 , 0.02% sodium

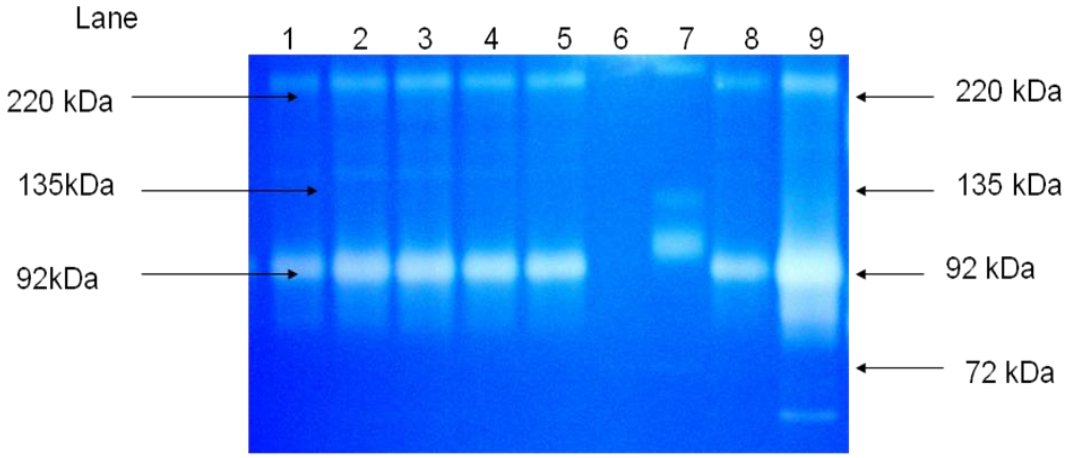
azide and 0.5 M α-methyl manno pyranoside). Aliquots of the eluents were subjected to SDS-PAGE and gelatin zymography.



(Lane 1-6 : Gelatin sepharose eluents E1, E2, E3, E4, E5 and E6. Each 10μl of fractions collected)

Figure 3: Gelatin sepharose chromatography of matrix metalloproteinases from canine mammary tumor





Lane 1-6 &8: Con-A sepharose eluents

Lane7 : Human Marker

Lane 9 : Gelatin sepharose eluent

Similar results : Murphy *et al*, 1989 : Hibbs *et al*, 1985)

Figure 4: Gelatin sepharose chromatography of matrix metalloproteinases from canine mammary tumor

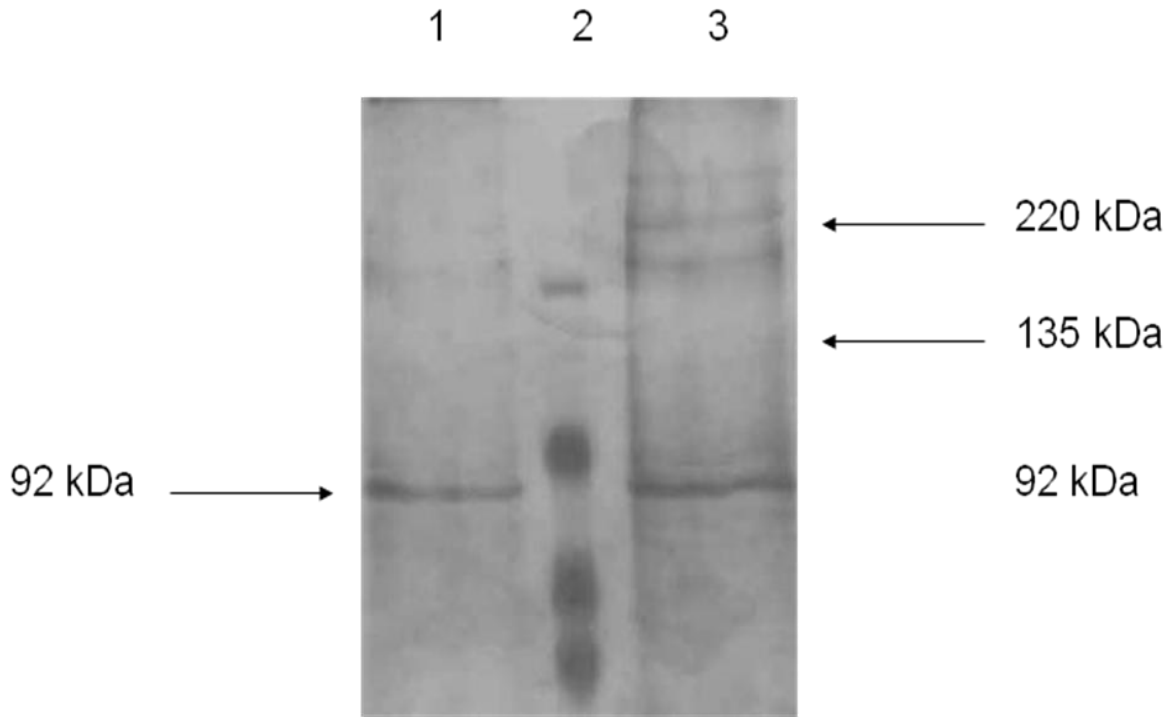


Figure 5: SDS-PAGE of Gelatin sepharose chromatography of matrix metalloproteinases from canine mammary tumor

Figure 6: Western blot of Gelatin sepharose chromatography of matrix metalloproteinases from canine mammary tumor

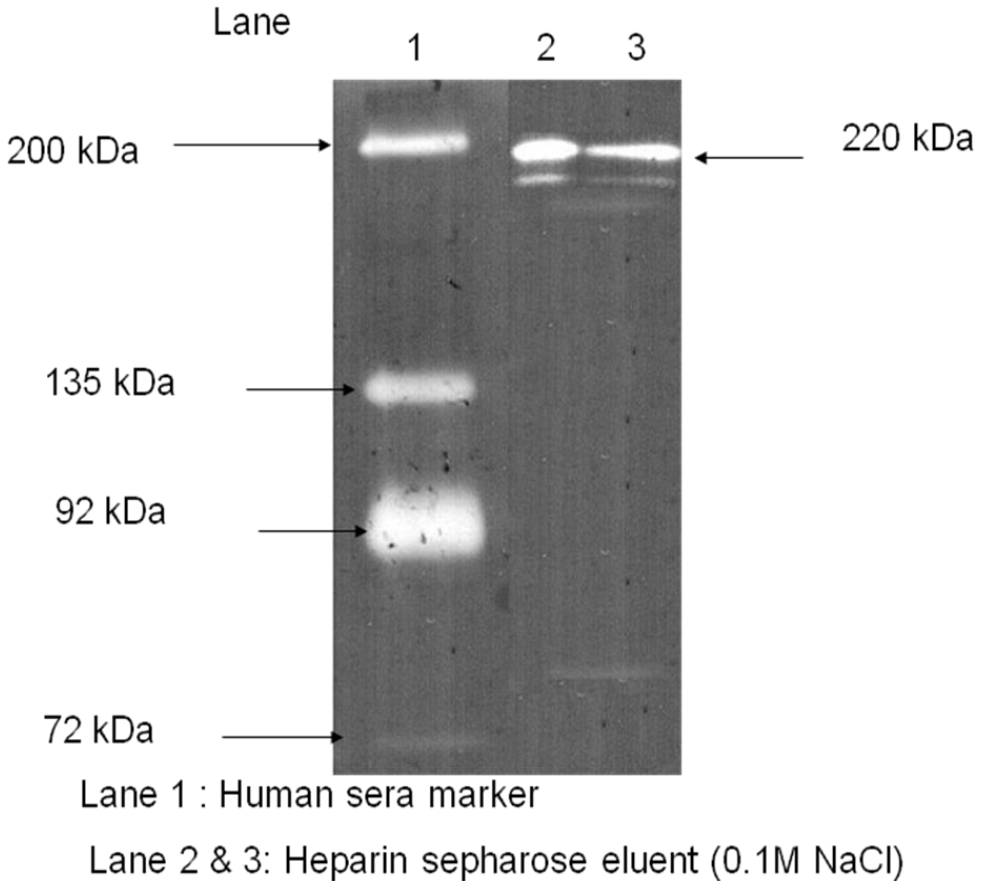


Figure 7: Gelatin zymography of heparin sepharose chromatography of matrix metalloproteinases from canine mammary tumor

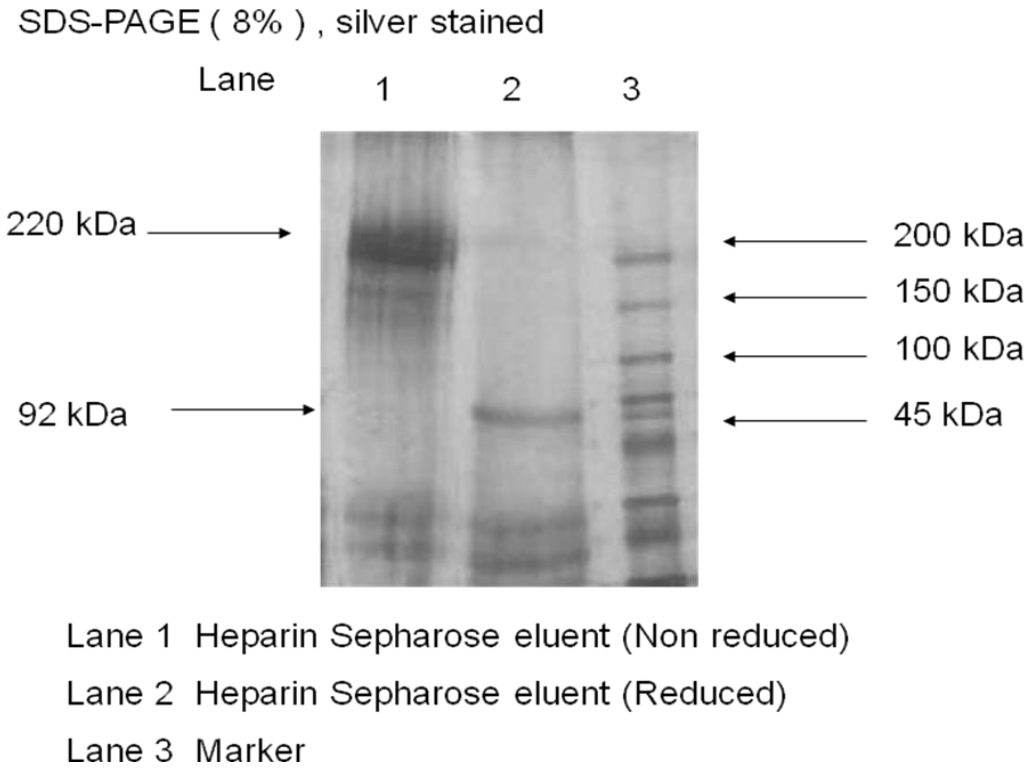


Figure 8: SDS-PAGE of heparin sepharose chromatography of matrix metalloproteinases from canine mammary tumor

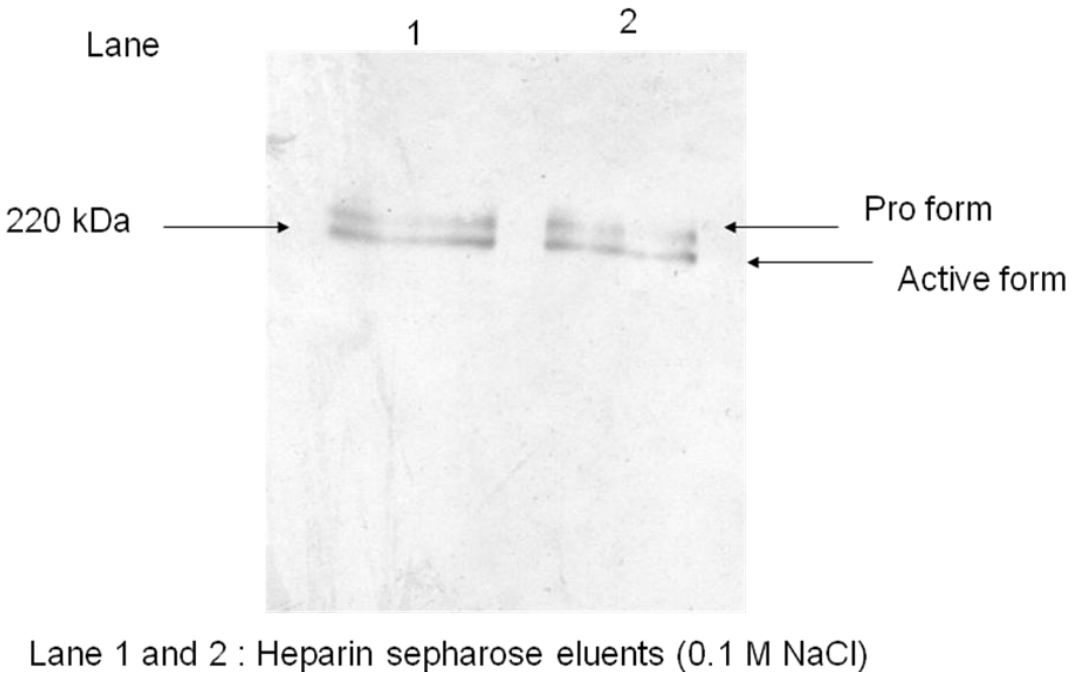


Figure 9: Western blot of heparin sepharose chromatography of matrix metalloproteinases from canine mammary tumor

Heparin sepharose chromatography of MMP-9

The method of Kolkenbrock *et al.*, (1991) was followed with some modifications. The eluents, of con-A sephrose chromatography were pooled and subjected to dialysis overnight with against 200 ml of equilibration buffer of Heparin sepharose chromatography(0.02 M Tris-Cl buffer, pH 8.0 containing 0.005 M CaCl2, 0.05% sodium azide and 0.02% Brij-35). 3 ml of heparin sepharose (sigma) was packed with 50 ml of equilibration buffer . The dialyzed sample was collected and applied to the heparin sepharose column at a flow rate of 10 ml/hour. Then, the column was washed with equilibration buffer, until the absorbance reached the baseline. Then, the bound MMP-9 was eluted, first with 10 ml of equilibration buffer containing 100 mM NaCl followed by another 10 ml of equilibration containing 200 mM NaCl . One ml fractions of eluents were collected in eppendorf tubes and absorbance were taken at 260 and 280 nm and the values were plotted against fraction volume. Aliquots from each fraction were subjected to SDS-PAGE and gelatin zymography, as described earlier.

Table 1: Purification summary of canine mammary tumor matrix metalloproteinases

Step	Total Proteins (mg)	Total Activity* (units)	Specific Activity (units/mg)	Purification (Fold)	Yield (%)
Tissue Homogenate	14980	18579	1.24	-	-
Gelatin Sepharose	2.5881	4751.65	1835.96	1480.61	25.56 (100%)
Con–A Sepharose	1.145	3012.09	2630.65	2121.49	16.21 (63.42%)
Heparin Sepharose	0.312	1165.34	3735.05	3012.14	6.21 (24.30%)

*One Unit refers to degradation of 1cm^2 area in gelatin Co-polymerized SDS-PAGE (gelatin Zymogram)
•Values given in the parentheses denote the yield obtained by keeping the gelatin sepharose chromatography eluents as the starting material

(similar results: Lana *et al.*, 2000, Loukopoulos *et al.*, 2003: Mandal *et al.*, 2003)

Determination of Total Mmp-9 Activity in Gelatin Zymography

Total MMP-9 activity was determined by the ability of the enzyme to degrade a known amount of gelatin co-polymerized with the resolving gel in SDS-PAGE. 150 mg% final concentration of gelatin was used in the resolving gel. The actual amount of gelatin present in the resolving gel could be determined by multiplying the concentration of the gelatin solution used by the volume of the gelatin solution taken in the resolving gel. From the total amount of gelatin present in the resolving gel, amount of gelatin present in q square centimetre of the gel could be calculated by dividing the total amount of gelatin present in the resolving gel by the total area of the resolving gel. When the MMP-9 present in the sample was made to separate in SDS-PAGE and subsequently renatured and developed, the gelatin present in the resolving gel

was degraded by the different forms of MMP-9 viz., 92 kDa, 135 kDa, and 220 kDa forms. This degradation of gelatin resulted in a clear zone visible on staining with Coomassie blue. The total area, degraded by individual forms of MMP-9 was individually measured in centimetres. From the area degraded by the individual enzyme, total amount of gelatin degraded by the individual enzyme could be calculated, then, total activity of MMP-9 was determined by summing up of the individual enzyme activities and multiplying the value by the total volume of the sample taken and the dilution factor.

Preparation of Polyclonal Antibodies against Canine Mmp-9 and Immunoblotting

The solutions containing MMP-9 activity were pooled from the gelatin sepharose chromatography and lyophilized at -80^{0}C. 600 mg of the lyophilized, purified MMP-9 from canine mammary tumor tissue was subjected to SDS-PAGE on a preparative 6% polyacryloamide gel. The Coomassie brilliant blue stained protein bands were identified as MMP-9 with help of human capillary blood MMP-9 used as standard. The MMP-9 bands were3 cut from the gel and homogenized in a buffer solution of 0.05 M Tric-HCl pH 7.6 containing 0.15 M NaCl and 0.08% SDS. The homogenized material was centrifuged at 12000 rpn and the supernatant was collected and used for immunization. 0.5 ml of the clear homogenoate was emulsified with an equal volume of complete Freund's adjuvant and injected intradermally into Newzealand white rabbits. Three boosters were given with Freund's incomplete adjuvant on day 14, 21 and 30th day of first injection. Test bleeding was collected on 42nd day and the presence of antibody was checked by AGPT. Then, the blood was collected from heart venepuncture of rabbits and serum was separated and used as polyclonal serum for ELISA and western blotting.

Immunoblotting

Immunoblotting wa carried out by the method suggested by Tgowbin *et al.*, (1979). The samples collected from the three chromatographic processes were subjected to SDS-PAGE, as described earlier. Then, the gel containing the separated proteins was equilibrated with transfer buffer for 5 minutes. Then, transfer of proteins to the 0.45µm Nitrocellulose membrane (Sigma) was carried out in 1x transfer buffer (0.025 M Tris, 0.192 M glycine pH 8.3 and 20% methanol) at 210 mA current for a period of two and half hours. Then, the membrane was put in 5% skimmed milk powder solution at 37 OC for 2 hours with gentle constant agitation. 3 to 4 washings were given with each 25 ml of PBS- Tween (137 mM NaCl, 2.7 mM KCl, 100 mM Na2HPO4, 2 mM KH2PO4 and 0.05 Tween 20, pH 7.4) in a period of 30 minutes. Primary Antibody (antibody developed in rabbits against gelatin sepharose eluents containing MMP-9) was applied at 1:2000 concentrations to the membrane and incubated overnight at 4OC. Then, washing with PBS was repeated. Secondary antibody at 1:2000 dilutions of goat anti-rabbit immunoglobulins fractions were applied to the membrane and incubated for one hour at 37OC with gentle and constant agitation. Then, washing with PBS was repeated. Rabbit anti-goat –alkaline phophatase conjugate at a dilution of 1:2000 was added and incubated for one hour at 37OC with gentle agitation. Washing process was repeated. Then substrate buffer(0.1M Tris pH 9.5, 0.1 M NaCl and MgCl2) containg 44 µl of 5% NBT and 88 µl of 5% BCIP was applied and the bands were allowed to develop and the reaction was stopped by adding distilled water. The membranes were air dried and stored at -20OC for future use.

Indirect Elisa of Mmp-9 in Sera and Tissue Homogenoate Samples Collected From Dogs Affected With Mammary Tumor

The method suggested by Perlman and Engvall (1971) was followed with minor modifications. Optimum concentrations of conjugate and developing reagent were determined by criss-cross dilution analysis. 50µl of the serum and tissue homogenate samples were diluted optimally in coating buffer (0.05 M carbonate-bicarbonate buffer pH 9.6) and incubated at 4^{0}C overnight in a humidified chamber. After washing the plates four times with PBS containing 0.05% Tween-20, blocking of unbound sites were carried out with 5% skimmed milk powder in PBS-T at 37^{0}C for 3 hours with mild agitation. Washing procedures were repeated. The plates were incubated for 2 hours with appropriate dilutions of HRPO-conjugate antirabbit immunoglobuins (50µl/well). Unbound conjugates were washed thoroughly with PBS-T and the colour reaction was developing with O-Phenyl diamine at 37^{0}C for 15 minutes. The reaction was stopped by adding 50µl of 3 M sulphuric acid to each well. The intensity of colour reaction was read at 492 nm using

an ELISA reader. The human standard MMP-9 (sigma) was diluted serially and the absorbance was read at 492 nm.

Biochemical Characterization of 220 Kda Mmp-9 Purified From Canine Mammary Tumor Tissue

Activation of Mmp-9 by 4-Aminophenyl Mercuric Acetate

50 µg of the purified MMP-9 was added to 1 mM APMA (50µl) in tissue homogenoate buffer and mixed and incubated at 37OC for 16 hours and then the aliquots from each tube were subjected to gelatin zymography.

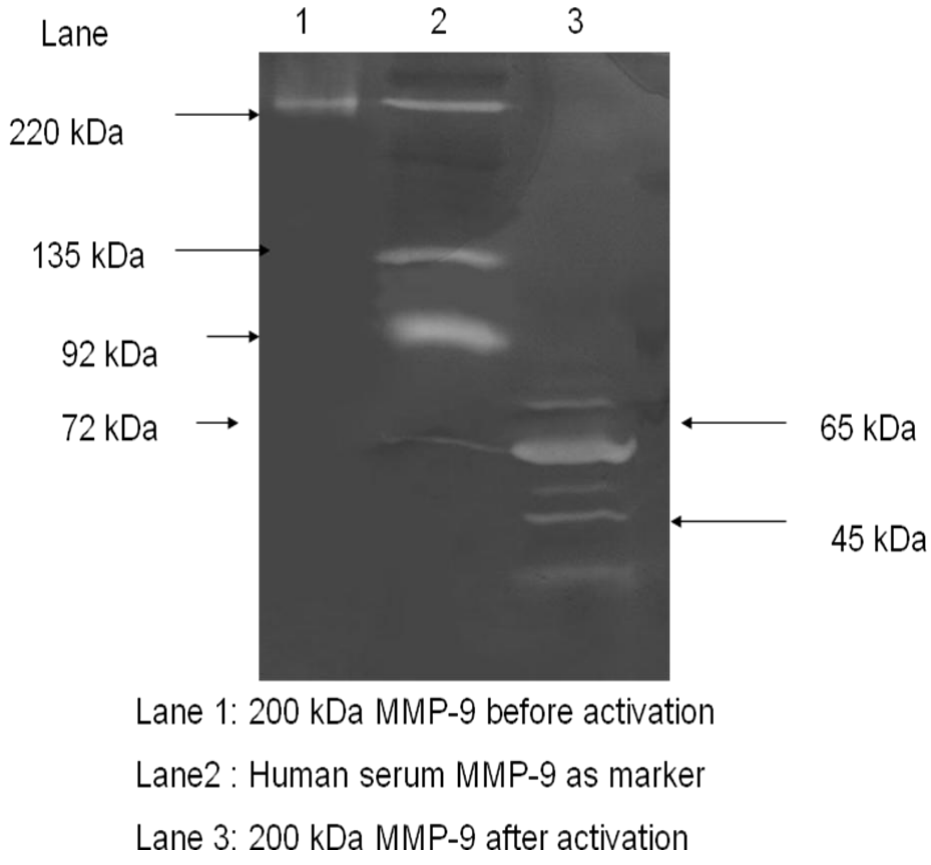


Figure 10: Activation of MMP-9 by 4-aminophenyl mercuric acetate

Effects of Inhibitors on Mmp-9 Activity

EDTA, 1,10-phenanthroline (1mM), 1,4-dithiothreitol (1mM), PMSF(2mM) and ß-mercaptoethanol (0.25%) were prepared in developing buffer of gelatin zymography. 6 eppendorf tubes containing each 20µg of purified MMP-9 were taken and 20 µl of each inhibitor was added to individual tubes and then the tubes were incubated at 37OC for a period of 16 hours. Then, aliquots from each tube were subjected to gelatin zymography on 8% resolving gel co-polymerized with gelatin and observed for the effect of inhibitors on MMP-9 activity.

RESULTS

Gelatin Sepharose, Con-A Sepharose and Heparin Sepharose Chromatograpy of Canine Mammary Tumor Tissue

The purification summary of canine mammary tumor tissue homogenoate was given in table 1. The prominent bands of 220 kDa, 92 kDa and 135 kDa bands of MMP-9 and a 72 kDa band of MMP-2 were

observed in gelatin zymography of the eluents collected from gelatin sepharose chromatography (fig. 1). The results of gelain zymography indicated that all the three forms of MMP-9 contained gelatinolytic activity, eliminating the possibility that one or more of the bands represented and inactive subunit or carrier protein. MMP-9 in its 92 kDa form was shown to be more expressed in malignant cases. The ratio of MMP-9/MMP-2 was higher in all the eluents. Among the three forms of MMP-9, the 92 kDa monomeric form was very prominent. When the same aliquots were subjected to SDS-PAGE (non-reducing, denaturing, Lammeli method), 220 kDa, 92 kDa and the 72 kDa bands were prominently observed (fig. 2). Under reducing conditions, only 92 kDa and 72 kDa bands were observed, In western blotting, all the three forms of MMP-9(220kDaa, 135kDa and 92 kDa) could be detected using both the anti-human MMP-9 antibodies and atni-canine MMP-9 220 kD antibodies (fig. 3).

In con-A sepharose chromatography eluents, peak elution was observed in fifth elution (each 1 mal eluents) (fig. 4). On gelatin zymography of these eluents, three major bands of MMP-9 (220 kDa, 135 kDa and 92 kDa) were observed, The 72 kDa MMP-2 band was totally eliminated out (fig. 5). SDS-PAGE of con-An eluents showed that the relative molecular mass of MMP-9 was estimated to be 220 kDa, 135 kDa and 92 kDa under non-reducing conditions (fig. 6). And a prominent 92 kDa band and a fainter 35 kDa bands were observed under reducing conditions (fig. 7). Immunoblot of these eluents revealed all the three forms of MMP-9.

In heparin sepharose chromatography, when the bound proteins were eluted with 0.1M NaCl and 0.2 M NaCl, two peaks were observed (fig. 8). In gelatin zymography, the 0.1 M NaCl eluted fractions showed only 220 kDa band of MMP-9. Both pro and active forms of 220 kDA MMP-9 were observed as two bands at 220 kDa region (fig. 9). When, the same samples were subjected to SDS-PAGE, a thicker 220 kDa band was observed under non-reducing conditions and 92 kDa band was observed under non-reducing conditions and the 92 kDa and a fainter 35 kDa bands were observed under reducing condions (fig. 10)a. The pro and active forms of the 220 kDa form of MMP-9 was detected on western blotting (fig 11) as doublets with anti-MMP-9 antibody.

Biochemical Characterization of 220 KDA Mmp-9 Purified From Canine Mammary Tumor Tissue

On incubation with APMA, the 220 kDa form of MMP-9 isolated from canine tumor tissue was converted into smaller catalytic fragments of size ranging from 65 kDa to 45 kDa. The smaller fragments still posed the ability to degrade gelatin substrate, as revealed through gelatin zymography (fig. 12). The 220 kDa form of MMP-9 was totally converted to smaller fragments and the 220 kDa activities were totally lost after the incubation time.

10 mM EDTA and 1 mM 1, 10–phenanthroline completely inhibited the MMP-9 activity on gelatin zymography. PMSF exerted least inhibitory action on the MMP-9 activity.

The optimum MMP-9 activity was found in a pH of 7.2 to 7.77 and the lowest activity was found in pH of 5.9 and 9.1.

The MMP-9 activity was found to be optimum with 10 mM CaCl2 and no activity was observed when no calcium chloride was added to the developing buffer of gelatin zymography.

Immunological Characterization of 220 KDA Form Of Mmp-9

The hyper immune serum raised form the rabbits injected with 220 kDa form of MMP-9 purified from canine tumor tissue detected all the three forms of MMP-9 (fig. 13) on western blot. In addition, all the three forms of canine MMP-9 were detected by anti-human MMP-9 antibodies. The presence of pro and active forms of the purified 220 kDa form of MMP-9 were observed on immunoblot by using anti-canine 220 kDa MMP-9 antibodies raised in rabbits.

Indirect ELISA of MMP-9

The linearity of the curve was maintained up to 1000 ng of 220 kDa form of MMP-9 isolated from canine mammary tumor. And the sensitivity of the test was 100 ng/ml.

The mean level of MMP-9 in normal dog serum samples was 340.9 ng/ml and in tuor dog sera, it was 728.656 ng/ml. The mean level of MMP-9 in the normal surrounding tissue was 118.61 ng/g tissues and

in tumor tissue, it was 768.23 ng/g tissue. There were 2.137 fold increases in the sera level of MMP-9 and 6.48 fold increase in the tissue level of MMP-9 between tumor and normal tissue of dogs (fig. 14).

DISCUSSION

Gelatin zymograpy of canine tumor tissue homogenoate revealed four prominent bands, 72 kDa, 92 kDa, 135 kDa and 220 kDA. Similar bands of 225, 120, 1000, 88, 62, 59 and 57 kDa and fainter bands at 42 kDa and 36 kDa in gelatin zymography have been earlier reported by Coughlan and Co-workers (1998b). Gelatin zymography could be used to identify the pr4esenece and amount of MMP-9 in the clinical samples collected from dogs. Both pro and active forms of MMP-9 were detected at a difference of 10 kDa. Similar results were already reported by Birkadel-Hanesen (1993) for MMP-9 in human tumor cases. The latent form of MMP-9 also displayed gelatinolytic activity because of the artificial condition of the zymographic process. However, only MMP-9 and MMP-2 can be measured by the gelain zymographic technique. Similar observations were reported by Parson et al., 1997 in human cancer samples. In our study, the level of expression of 92 kDa form of MMP-9 was found to higher in tumor bearing dogs than in the normal dogs. The expression of MMP-9 in benign tumor cases was higher than in the normal mammry tissue and it was much higher in malignant mammry tumor of dogs. Similar results have already been reported by Hirayama *et al.,* 2002. In the present study, the MMP-9 was found to be present in 3 different forms, 92, 135 and 220 kDa forms, by comparing them to the human capillary blood MMP-9 as standard. Coughlan *et al.,* (1998a) reported that the 75 kDa is the active form of canine MMP-9 and 88 kDa is the monomeric latent form of MMP-9 (proMMP-9) in dogs. Loukopoulas *et al.,* 2002 reported the 92 Da of MMP-9 were found in pro and active form at a differnce of 10 kDa. The smaller difference in the sizes reported by earlier authors could be related to the degree of glycosylation of MMP-9 in different breeds.

In SDS-PAGE, three proteins bands of Mr 220, 135 and 92 kDa were observed under non-reducing conditions and only one band of Mr at 92 kDa were observed, suggesting all the three bands were related and it was likely that the 92 kDa form may represent the basic subunit of the enzyme. Such similar results were reported by Tirebel *et al.,* (1992a) in case of PMNL type IV procollagenae as 220 kDa, 125 kDa and 98 kDa and under reducing conditions, as 98 kDa and 28 kDa bands.

The 220 kDa form of MMP-9 which was isolated in the present study could be a dimeric form of 92 kDa monomeric form of MMP-9, as the 220 kDa was converted into 92 kDa, as observed in reducing conditions of SDS-PAGE. Further, the 220 kDa form was totally converted into smaller catalytic subunits by APMA treatment. The 220 kDa form of MMP-9 was identified in both normal and tumor tissue and also in the serum of both normal and tumor bearing dogs, indicating that 220 kDa form is the physiological form of the enzyme. Both the dimer form (220kDa) and the monomeric for (92kDa) were catalytically active, as revealed in gelatin zymography. Olson *et al.,* (2000) reported that the 92 kDa monomeric and 220 kDa dimeric form of MMP-9 cleared the gelatin substrate with similar catalytic activity.

The expression level of MMP-2 was found to be constant in both the diseased and normal dog sera samples suggesting the constitutive synthesis of MMP-2. Birkadel-Hansen et al 1983 reported that 72 kDa MMP-2 was expressed constitutively by most cell type in vitro and was also present in the normal human plasma at a concentration of 350 to 500 ng/ml. MMP-9 was found to be increased with the tumor bearing dog sera samples than in normal sera. As a whole, the level of MMP-9/MMP-2 was higher in sera samples of tumor bearing dogs than the normal ones.

Using gelatin sepharose chromatography, Coughlan *et al.,* (1998a) isolated MMP-2 and MMP-9 from myelomonocytic cell lines, Hibbs *et al.,* (1985) and Wilhelm *et al.,* (1989) utilized the on step procedure of gelatin sepharose chromatography for purifying human MMP-9 and MMP-2. In the present study, using gelatin sepharose chromatography, all the three forms of MMP-9 (92 kDa, 135 kDa and 220 kDa) and MMP-2 (72 kDa) were isolated from the tissue homogenoate. Using Con-A sepharose chromatography, the glycosylated MMP-9 was isolated from the non-glycosylated MMP-2, as MMP-2

could not bind to Con-A sepharose. The glycosylated MMP-9 was bound to the con-A sepharose and then could be eluted by α-methyl manno pyranoside.

In heparin sepharose chromatography, the different salt concentration used in the elution buffer helped to isolate the 220 kDa form of MMP-9 from the other two forms (135 and 92 kDa). Such isolation of isoforms of MMP-9 has already been reported by Kolkenbrock *et al.,* (1997) in human tumor cases.

Activation of the 220 kDa MMP-9 was achieved by 1 mM APMA and the activity of the enzyme was totally absent , if no calcium ions were added to the developing buffer of gelatin zymography and the activity of the enzyme was inhibited completely by 1,10-phenanthroline and EDTA. The optimum pH for the activity of 220 kDa MMP-9 was 7.4. All these facts suggested that this enzyme is a metalloproteinase, requiring divalent cation for its activity.

In the present study, there was 2.137 fold and 6.48 fold increase in the MMP-9 level of sera and the tumor tissue of dog affected w9ith mammary tumor, respectively. This could be attributed to the infiltration of the enzyme and its concentration at the tumor site, may be an inflammatory response. Such infiltration of the PMNL at the tumor site and increase in the tissue leel of MMP-9 in tumor cases of human beings was reported by Birkadel-Hansen1993.

The antibodies raised against 220 kDa form of canine MMP-9 in rabbits detected all the three forms of MMP-9 (92, 135 and 220 kDa) in western blotting, suggesting all the three forms shared basic structure. The canine MMP-9 enzyme was detected by the antibodies raised against human MMP-9 suggesting that the cross-reactivity and sharting of at least some common epitopes among these two species of enzymes.

Taken together, these results conclusively indicated that the purified enzyme is canine tumor tissue MMP-9 according to the following criteria: Molecular weight, specific inhibition by EDTA, activation by APMA, binding to gelatin matrix and immuno reactivity and complete dependence on divalent cations for its activity. These studies suggested that MMP-9 might not only be good targets for antineoplastic therapy but may have clinicala utility in identifying the subgroups of patients at increased risk of recurrence and metastasis. Although most studies have quantified the total enzyme level and correlated to the tumor invasiveness, the amount of active form of enzyme present in a tumor sample may be important than evaluating the total enzyme. Future trials may be designed to take advantage of the prognostic information provided by these markers of biological aggressiveness.

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