International Journal of Food, Agriculture and Veterinary Sciences ISSN: 2277-209X (Online)
An Online International Journal Available at http://www.cibtech.org/jfav.htm
2013 Vol. 3 (1) January-April, pp. 164-176/ Krupakaran
Research Article

ISOLATION AND CHARACTERIZATION OF NEUTROPHIL MATRIX METALLO –PROTEINASE-9 (MMP-9) FROM CANINE MAMMARY TUMOR

[*]R. Prakash Krupakaran
Department of Veterinary Physiology and Biochemistry,
Veterinary College and Research Institute, Orathanadu, Thanjavur District-614625
**Author for Correspondence*

ABSTRACT
Matrix metalloproteinase-9 (MMP-9) was isolated and purified from neutrophils isolated from canine blood samples, by adopting stepwise chromatographic procedures: gelatin sepharose, con-A sepharose and heparin sepharose. In gelatin sepharose chromatography, both MMP-9 and MMP-2 were recovered to 9.19% from neutrophils. In con-A sepharose chromatography, MMP-9 was separated from MMP-2 and the yield was 6.50% for neutrophils. In heparin sepharose chromatography, 220 kDa form MMP-9 was isolated in 0.1 M NaCl elution from 135 and 92 kDa forms and the yield was 3.7% for neutrophils. SDS-PAGE revealed Mr 72, 92, 135 and 220 kDa bands under non-reducing conditions and Mr 92 kDa bands and 35 kDa bands under reducing conditions. Western blot revealed Mr 92, 135 and 220 kDa bands with anti-human MMP-9 antibody and Mr 92, 135 and 220 kDa bands with anti-canine antibody. Biochemical characteristics of isolated 220 kDa form of MMP-9 were studied. On activation with organomercurial compounds like 4- amino phenyl mercuric acetate (APMA), 220 kDa MMP-9 was converted into smaller forms of having molecular masses in the range of 45 and 65 kDa. Complete inhibition of the enzymatic activity was observed with EDTA and 1, 10-phenanthroline. 220 kDa form of MMP-9 activity was completely inhibited by 10 mM EDTA and 1mM 1, 10-phenanthroline.The activity of MMP-9 (220 kDa) was totally dependent on the presence of divalent cation Ca2+. In the absence of calcium ions, total loss of enzyme activity was observed in gelatin zymography. The MMP-9 had an optimum pH of 7.2 -7.7 and optimum temperature of 37^{O}C-42^{O}C, for its fullest activity. The MMP-9 was found to be stable at -70^{O}C, for at least three months in the presence of 0.02% Brij-35. Addition of 10 mM monosaccharides to the developing buffer of gelatin zymography had no impact on the activity of MMP-9. The purified 220 kDa form of MMP-9 from canine tumor tissue 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). The level of MMP-9 was 2.137 fold higher in diseased sera and 6.48 fold higher in tumor tissue when compared to normal control. The level of MMP-9 in urine sample of tumor bearing dogs was found 18 fold higher than in normal dogs in tumor cases.

Key Words: *Matrix Metalloproteinase-9, Canine Mammary Tumor, Neutrophils, 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 behavior under physiological conditions (Dubois *et al.*, 2002). 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 Van den 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 tumor progression and metastasis (Stetler–Stevenson, 1996). Elevated production of MMP enzymes by cancer cells and tumor stroma correlates with the malignant and metastatic phenotype. But the exact location of the production of these enzymes is unknown. MMPs may

originate in tumor cell themselves or in nearby stromal components. There are several reports showing various sources of MMP-2 and MMP-9; tumor cells (Jeziorska *et al.,* 1996), stromal cells (Poulsom *et al.,* 1982), peripheral blood leukocytes (Murphy and Docherty, 1992) and inflammatory cells (Hibbs *et al.,* 1987).

The cellular source of MMP-9 in synovial fluids from dogs with rheumatoid arthritis is likely to be the large number of the recruited PMNL, which store MMP-9 in cytoplasm granules (Coughlan, 1998). Recent evidence suggested a role of inflammatory cells, which are a source of MMP-9 in cancer phenotype (Coussens *et al.,* 2000).

Masure and co-workers (1991) reported the isolation of 91 kDa active enzyme from human neutrophils and showed that the 91 kDa enzymes is a truncate form of tumor derived 92 kDa gelatinase, lacking 8 residues of the neutrophil gelatinase.

Tumours of mammary gland are the most common tumor of female dogs representing approximately (30-50%) of all tumours in bitch (O'Keefe, 1998). MMP activity in tumor tissue is higher than in unaffected stromal tissue, indicating the canine MMP-9 may be linked in pathogenesis of tumor growth and metastasis (Lana *et al.,* 2000).

As the dog is an important species both clinically and in studies of comparative pathology, it is important to identify and characterize the canine MMPs from different sources.

The present paper deals with isolation and characterization of MMP-9 from the neutrophils isolated from the blood samples collected from dogs affected with mammary tumor.

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 tumor and tissues surrounding the tumor. 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 hemotoxylin and eosin (H and 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 tumor 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.

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

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.

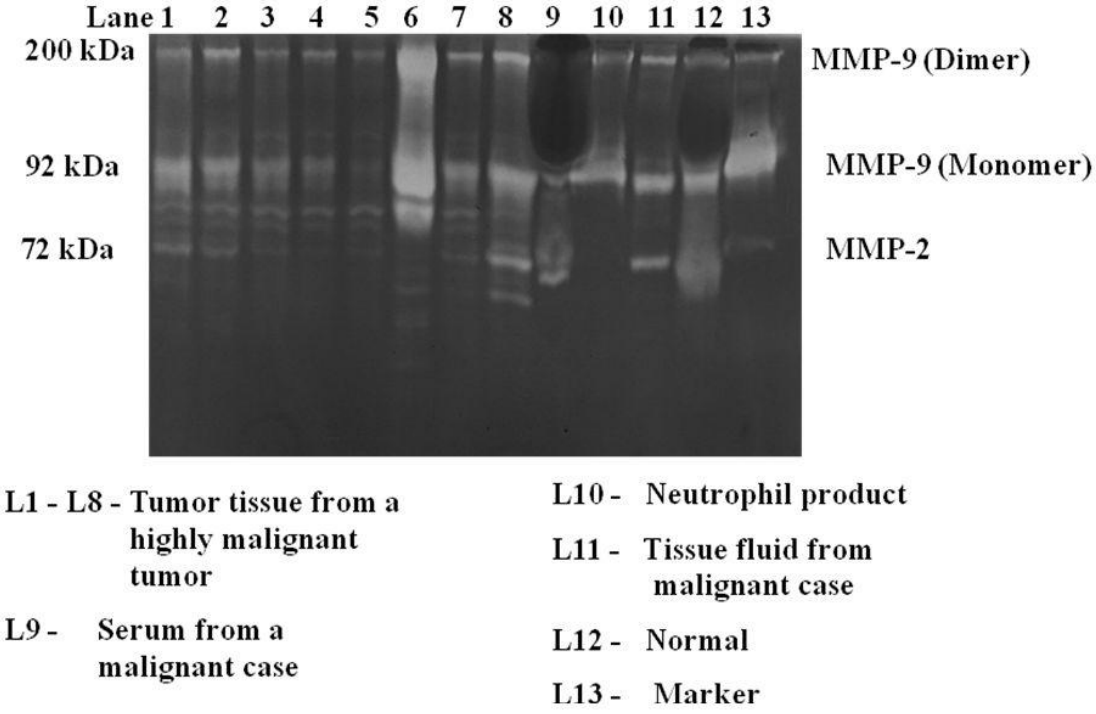

Figure 1: Gelatin zymograms of canine mammary tumor tissues

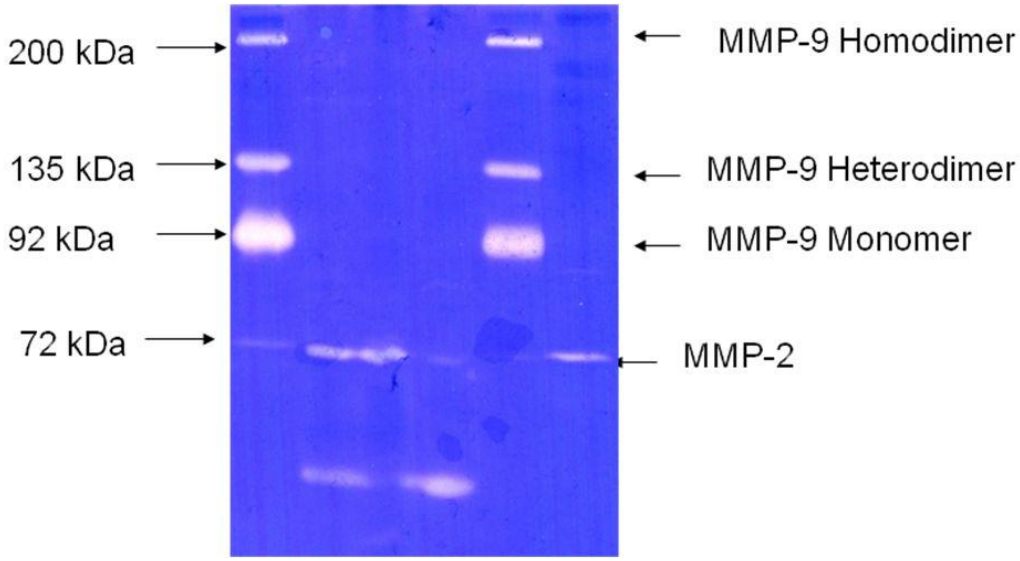

Figure 2: Calibrating gelatin zymograms with human capillary gelatinases

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^OC 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.

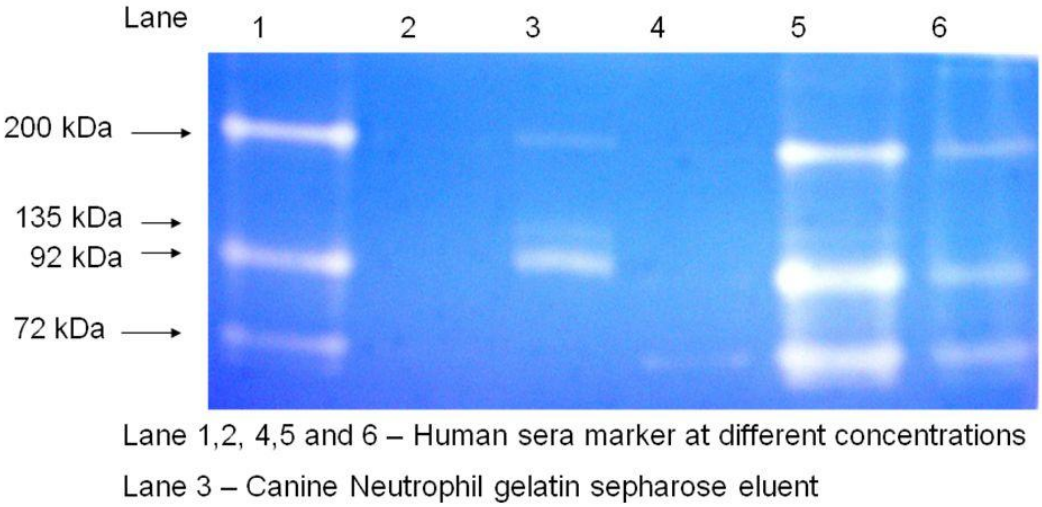

Figure 3: Gelatin zymography of Gelatin sepharose chromatography of matrix metalloproteinases from neutrophils of canine mammary tumor

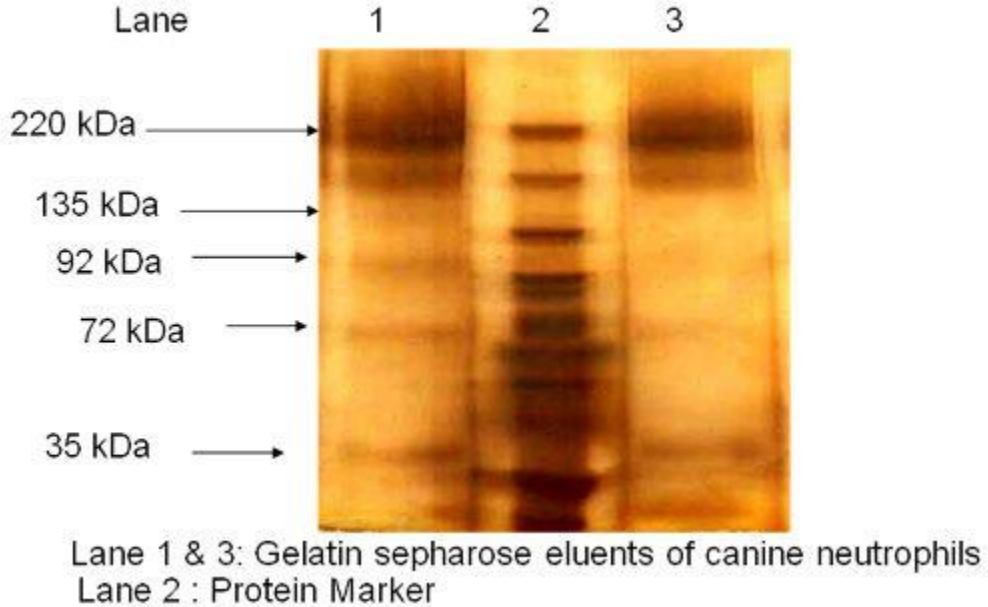

Figure 4: SDS-PAGE of Gelatin sepharose chromatography of matrix metalloproteinases of canine mammary tumor neutrophils

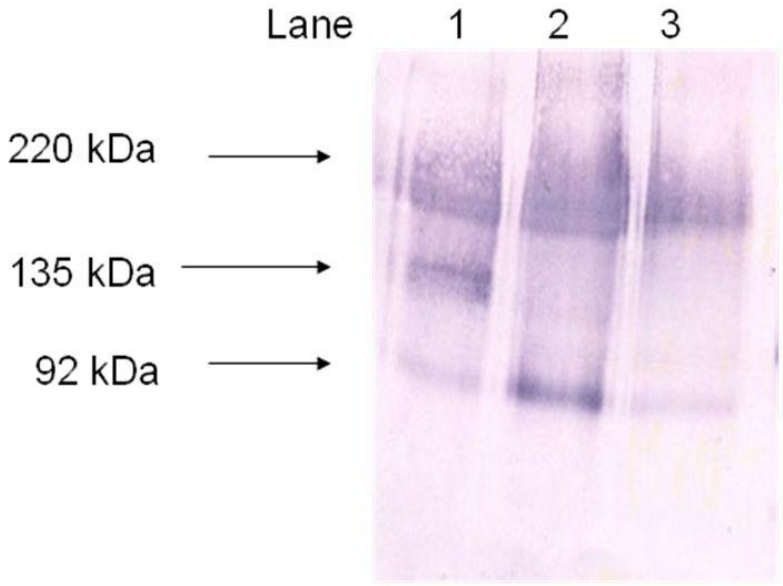

Figure 5: Western blot of Gelatin sepharose chromatography of matrix metalloproteinases of canine mammary tumor neutrophils

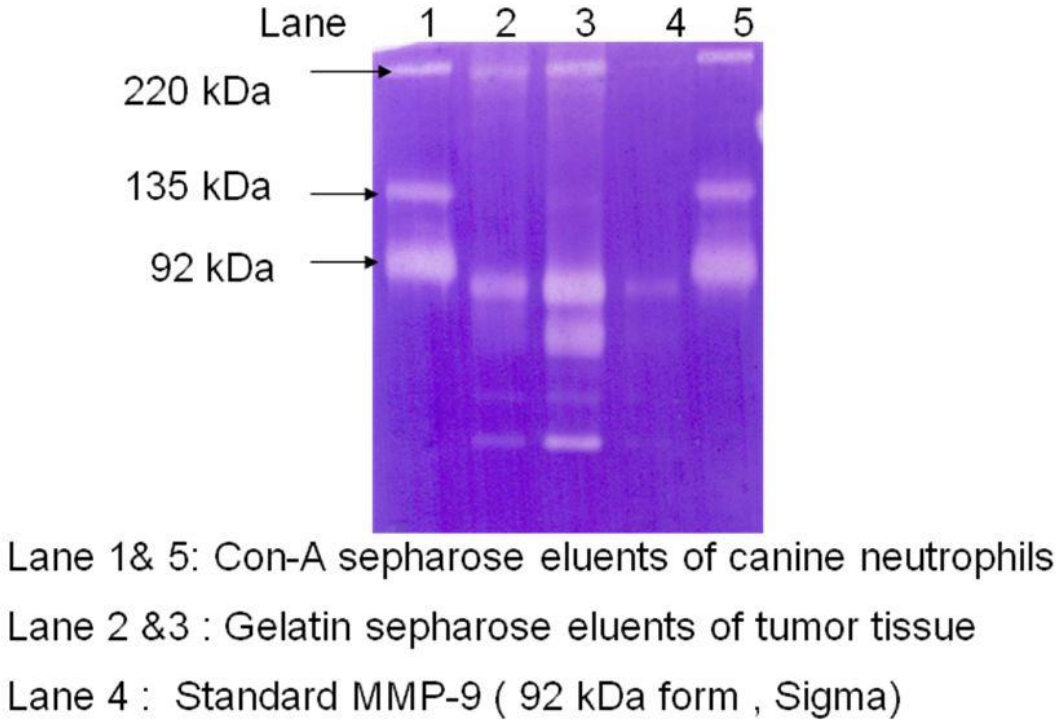

Figure 6: Gelatin zymography of Concanavalin- A sepharose chromatography of matrix metalloproteinases from neutrophils of canine mammary tumor

Concanavalin –A sepharose chromatography of MMP-9

The method suggested by Mandal *et al.*, (2003) was followed with some modifications. The eluents , showing gelatinase 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.

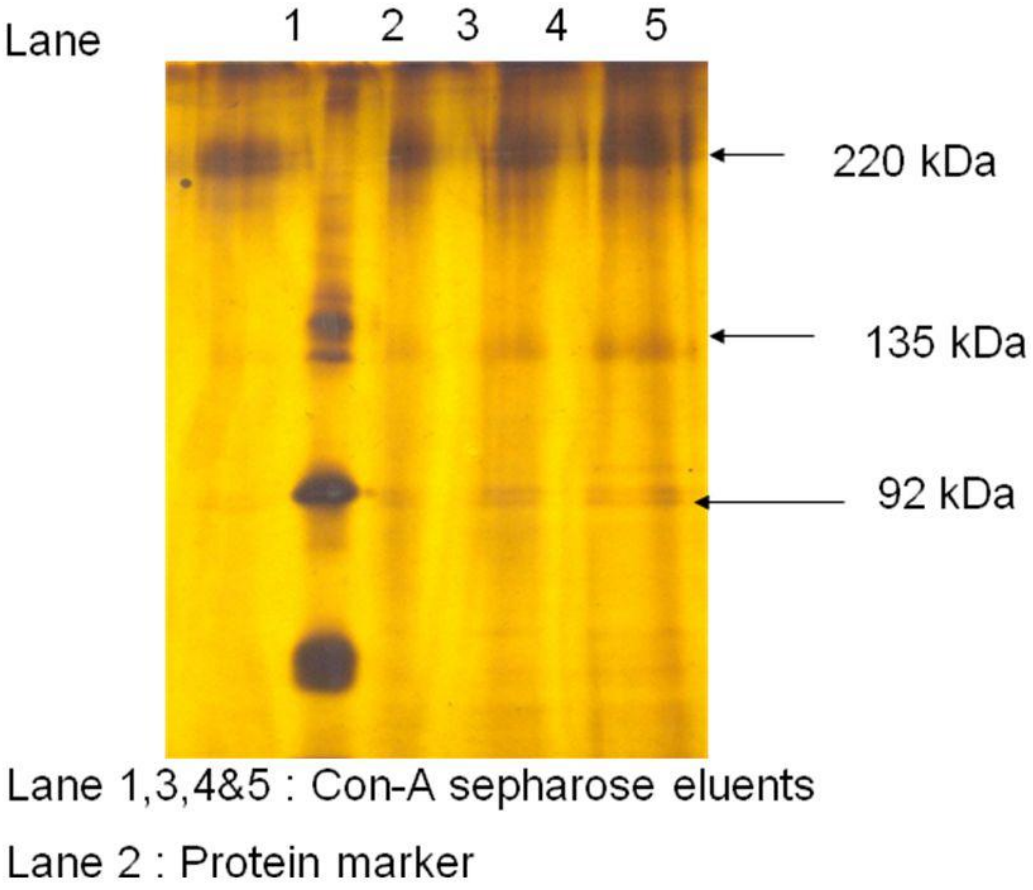

Figure 7: SDS-PAGE of Concanavalin-A sepharose chromatography of matrix metalloproteinases of canine mammary tumor neutrophils

Heparin sepharose chromatography of MMP-9

The method of Kolkenbrock *et al.*, (1991) was followed with some modifications. The eluents , of con-A sepharose 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 $CaCl_2$, 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 absorbances 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.

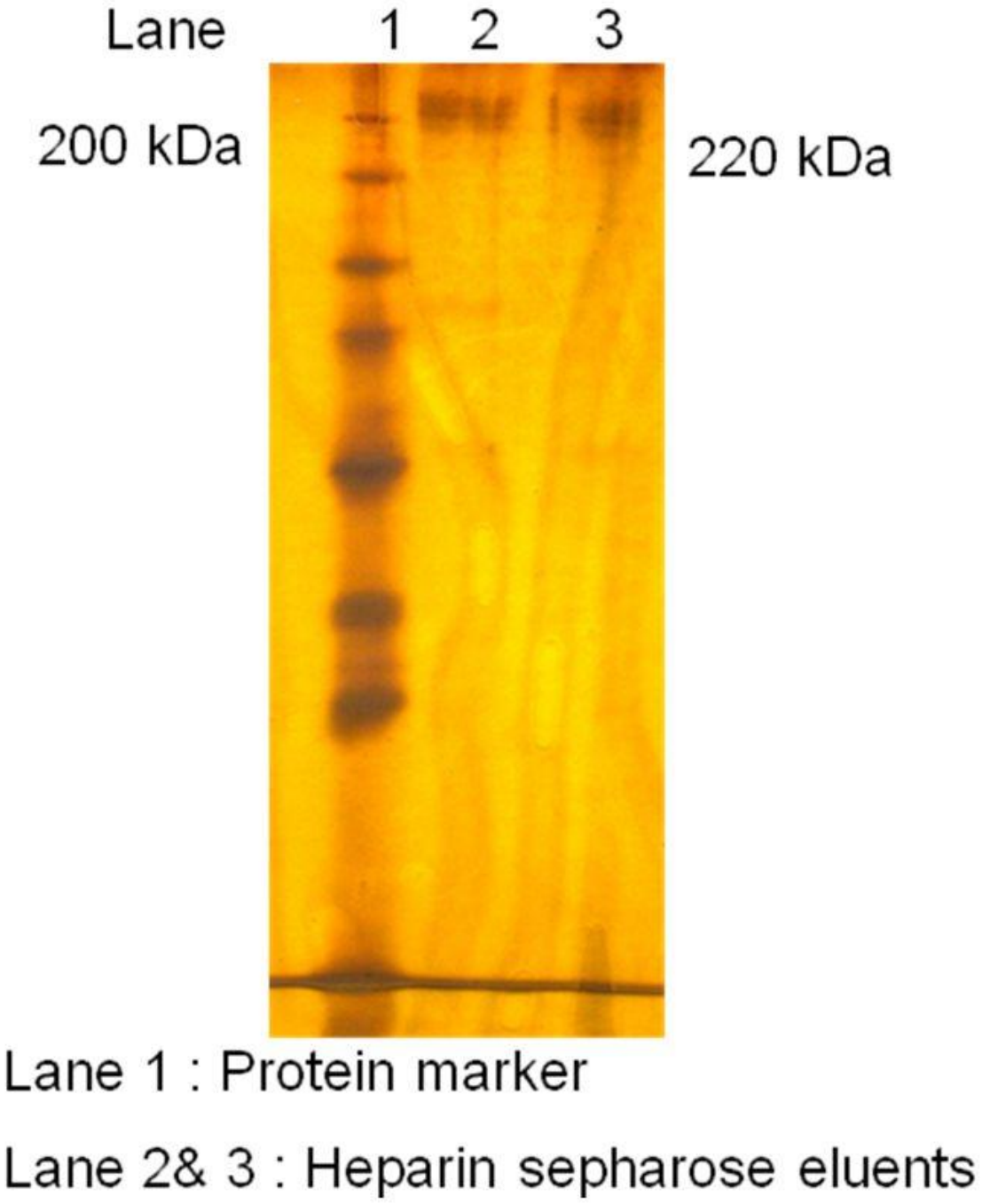

Figure 8: SDS-PAGE of heparin sepharose chromatography of matrix metalloproteinases of canine mammary tumor neutrophils

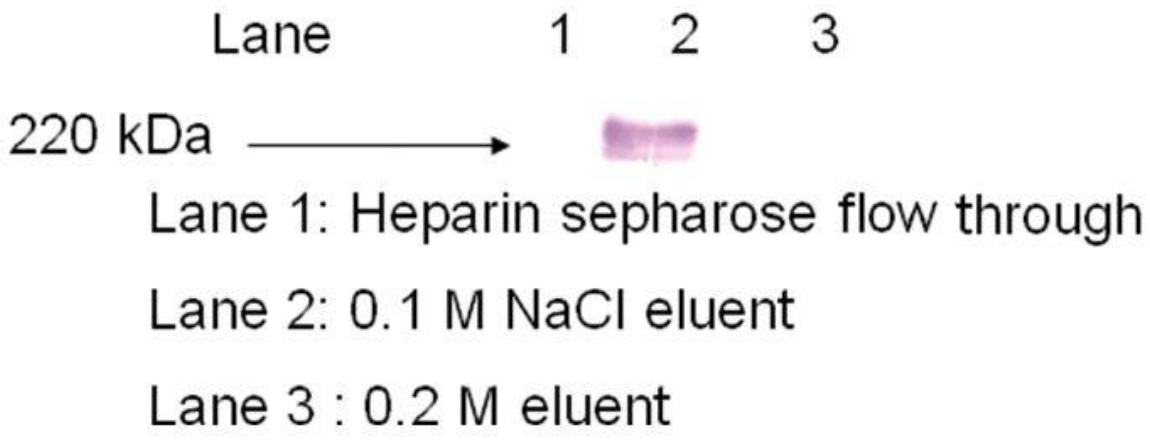

Figure 9: Western blot of heparin sepharose chromatography of matrix metalloproteinases of canine mammary tumor neutrophils

Step	Total Proteins (mg)	Total Activity* (units)	Specific Activity (units/mg)	Purification (Fold)	Yield (%)
Crude extract	55000	102300.6	1.86	-	-
Gelatin Sepharose	3.286	9398.95	2860.6	1537.98	9.19 (100%)
Con–A Sepharose	1.431	6646.71	4644.8	2497.20	6.50 (70.73%)
Heparin Sepharose	0.513	3654.71	7120.3	3828.19	3.57 (38.85%)

*One Unit refers to degradation of 1cm² area in gelatin Co-polymerized SDS-PAGE (gelatin Zymogram)
•Values in parentheses are the yield obtained starting from gelatin sepharose chromatography eluents
(Similar results : Hibbs *et al.*, 1985: Murphy *et al.*, 1989)

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 containg 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% polyacrylamide 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 Tris-HCl pH 7.6 containing 0.15 M NaCl and 0.08% SDS. The homogenized material was centrifuged at 12000 rpm 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 was carried out by the method suggested by Towbin *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 Na_2HPO_4, 2 mM KH_2PO_4 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 37^{O}C 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 $MgCl_2$) 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 -20^{O}C 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 homogenoate samples were diluted optimally in coating buffer (0.05 M carbonate-bicarbonate buffer pH 9.6) and incubated at 4OC overnight in a humidified chamber. After washing the plates four times with PBS containing 0.05% Tween-20, blocking of unbound sites was carried out with 5% skimmed milk powder in PBS-T at 37^{O}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 immunoglobulins (50µl/well). Unbound conjugates were washed thoroughly with PBS-T and the colour reaction was developing with O-Phenyl diamine at 37^{O}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.

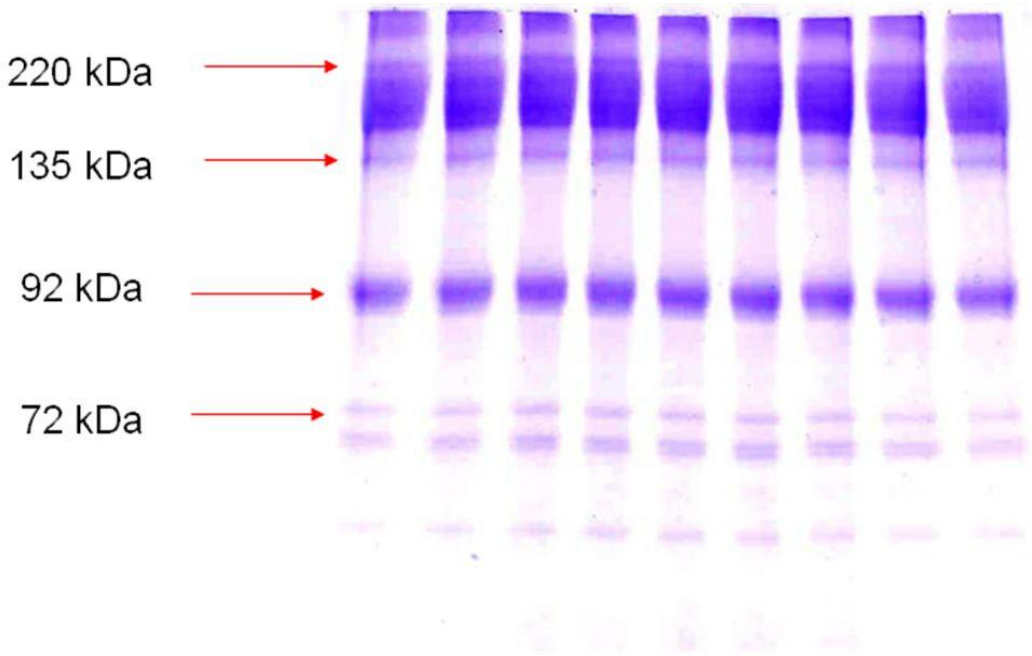

Figure 10: Preparative SDS-PAGE for the production of hyperimmune serum against MMP-9

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 37^{O}C for a period to 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 Chromatography 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. The results of gelatin zymography indicated that all the three forms of MMP-9 contained gelatinolytic activity, eliminating the possibility that one or more of the bands represented an 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,

Lamelli method,), 220 kDa, 92 kDa and the 72 kDa bands were prominently observed. 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 anti-canine MMP-9 220 kDa antibodies.

In con-A sepharose chromatography eluents, peak elution was observed in fifth elution (each 1 mal eluents). 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. 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. And a prominent 92 kDa band and a fainter 35 kDa bands were observed under reducing conditions. Immunoblotting 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. 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. When, the same samples were subjected to SDS-PAGE, a thicker 220 kDa band was observed under non-reducing conditions and 92 kDa bands was observed under non-reducing conditions and the 92 kDa and a fainter 35 kDa bands were observed under reducing conditions. The pro and active form of the 220 kDa form of MMP-9 was detected on western blotting 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 possessed the ability to degrade gelatin substrate, as revealed through gelatin zymography. 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 $CaCl_2$ 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 from the rabbits injected with 220 kDa form of MMP-9 purified from canine tumor tissue detected all the three forms of MMP-9. 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 immunoblotting 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 tumor 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 tissues. 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.

DISCUSSION

The isolation of MMP-9 from neutrophils has been reported earlier by several authors. Paeman *et al.,* (1995) purified gelatinase B (MMP-9) from human neutrophils on gelatin sepharose and reported three forms of gelatinase B (molecular mass of 97, 125 and 220 kDa) on non-reducing SDS-PAGE. Masure *et*

al., 1991 reported only two stainable protein bands of 91.2 kDa and 75.9 kDa from the eluents of neutrophil homogenoate on gelatin sepharose chromatography.

In the present study, 220, 135 and 92 kDa form of MMP-9 was found to be present in the crude extracts of canine neutrophils. The 72 kDa MMP-2 could not be observed. Kjeldesen et al, 1992 reported that 220, 135 and 92 kDa forms are the three forms of MMP-9 present in neutrophils and the 135 kDa form of gelatinase B (MMP-9) to be a complex of 92 kDa gelatinase and a 25 protein part and the 220 kDa form was the homodimer of the 92 kDa enzyme. Jeziorska and co-workers (1996) showed a clear association between the level of expression of gelatinase B and influx of polymorphonuclear leucocytes, macrophages and eosinophils in normal reproductive cycling. Over expression of proteases in the tumor cells and non-malignant (stromal-inflammatory) cells. Originally, gelatinase B was identified as a product of polymorphonuclear leucocytes and macrophages and its appearance to be part of the proteolytic arsenal used by these cells for extravastation from blood vessels and subsequent invasion into tissue.

The neutrophils (10^9 cells) contain approximately 0.2 mg of collagenase and 0.1 mg of gelatinase (Murphy *et al.,* 1982). Pyke and co-workers (1992) reported that the 72 kDa and 92 kDa metalloprote4inases are not necessarily produced by the malignant cell themselves, but may be generally by induction or recruitment of non-malignant stromal cells. These cells may interact with each other and thereby modify their function, such as expression of cell surface receptors or the release of inflammatory peptides. The fast release of metalloproteinase from leukocyte may allow these cells to solubilise the extracellular matrix as an aid to enter the tissues to reach the inflammatory focus in response to chemotactic factors. Ballin *et al.,* (1990) reported that the 92 kDa metalloproteinase of tumor cell is the counterpart of the neutrophil gelatinase. Makowski and Ramsby (1996) reported that MMP-9 was not constitutively produced by most cells and the recruitment of the inflammatory cells at the site of disease focus would provide increase in the enzyme activity. Coussens and co-workers (2000) reported that inflammatory cells are the critical suppliers of MMP-9 in the carcinogenesis pathway.

REFERENCES

Ballin M, Gomez DE, Sinha CC and Thorgeirsson UP (1998). *Tas*oncogene mediated induction of a 92 kDa metalloproteinase; strong correlation with the malignant phenotype. *Biochemical and Biophysical Research Communication* **154** 832-838.

Coussens LM, Tinkle CL, Hanahan D and Werb Z (2000). MMP-9 supplied by bone marrow – derived cells contributes to skin carcinogenesis. *Cell* **103** 481-490.

Coughlan AR, Robertson DHL, Burke R, Beynon RJ and Crater SD (1998). Isolation and identification of canine matrix metalloproteinase-2 (MMP-2). *Veterinary Journal* **155** 231-237**.**

Declerck YA (2000). Interactions between tumor cells and stromal cells and proteolytic modification of the extracellular matrix by metalloproteinases in cancer. *European Journal of Cancer* **36** 1258-1268.

Dubois B, Starckx S, Pagenstecher A, Oord JV, Arnol B and Opdenakker G (2002). Gelatinase B deficiency protects against endotoxin shock. *European Journal of Immunology* **32** 2163-2171.

Heussen C and Dowdle EB (1980). Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Analytical Biochemistry* **102** 196-202.

Hibbs MS, Hoidal JR and Kang AH (1987). Expression of a metalloproteinase that degrades native type V collagen and denatured collagens by cultured human alveolar macrophages. *Journal of Clinical Investigation* **80** 1644-1650.

Jayakumar R (1997). A pilot survey of the dog population and rabies vaccination in India. *Biomedicine* **17**(1) 25-27.

Jeziorska M, Nagase H, Salamonsen LA and Wolley DE (1996). Immunolocalization of the matrix metalloproteinases gelatinase B and stromelysin 1 in human endometrium throughout the menstrual cycle. *Journal of Reproduction and Fertility* **107** 43-51.

Kjeldsen L, Johnsen AH, Sengelov H and Borregarrd N (1992). Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *The Journal of Biological Chemistry* **268**(14) 10425-10432.

Lana SE, Ogilvie GK, Hansen RA, Powers BE, Dernell WS and Withrow SJ (2000). Identification of matrix metalloproteinases in canine neoplastic tissue. *American Journal of Veterinary Research* **61**(2) 111-114.

Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680-685.

Loukopoulos P, Mungall BA, Straw RC, Thornton JR and Robinson WF (2003). Matrix metalloproteinase-2 and -9 involvements in canine tumors.*Veterinary Pathology* **40** 382-394.

Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951). Protein measurement with the folin phenol reagent. *The Journal of Biological Chemistry* **193**(1) 265-275.

Mandal M, Das S, Chakraborti T, Mandal A and Chakraborti S (2003b). Identification, purification and partial characterization of tissue inhibitor of matrix metalloprotease-1 in bovine pulmonary artery smooth muscle. *Molecular and Cellular Biochemistry* **254** 145-155.

Makowski GS and Ramsby ML (1996). Calibrating gelatin zymograms with human gelatinase standards. *Analytical Biochemistry* **236** 353-356.

Masure S, Proost P, Van Damme J and Opdenakker G (1991). Purification and identification of 91-kDa neutrophil gelatinase. Release by the activating peptide interleukin-8. *European Journal of Biochemistry* **198** 391-398.

Merril CR, Goldman D, Sedman SA and Ebert MH (1981). Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. *Science* **211** 1437-1438.

Murphy G, Reynolds JJ, Bretz U and Bagiolini M (1982). Partial purification of collagenase and gelatinase from human polymorphonuclear leucocytes. *Biochemical Journal* **203** 209-221.

Murphy G and Docherty AJP (1992). The matrix metalloproteinases and their inhibitors. *American Journal of Respiratory Cell and Molecular Biology* **7** 120-125.

Nagase H and Woessner Jr JF (1999). Matrix metalloproteinases. *The Journal of Biological Chemistry* **274**(31) 21491-21494.

O' Keefe DA (1995). Tumours of genital system and mammary glands. In: Ettinger SJ (Edition), Textbook of veterinary internal medicine. *4th edition WB Saunders, Philadelphia* 1699-1703.

Paeman L, Matens E, Masure S and Opdenakker G (1995). Monoclonal antibodies specific for natural human neutrophil gelatinase B used for affinity purification, quantitation by two sites ELISA and inhibition of enzymatic activity. *European Journal of Biochemistry* **234** 759-765.

Perlman P and Engvall E (1971). Enzyme linked immunosorbent assay (ELISA): quantitative assay for immunoglogulin. *Immunochemistry* **8** 871.

Poulsom R, Pignatelli M, Steler –Stevenson WG, Liotta LA, Wright PA, Jeffery RE, Longcroft JM, Rogers L and Stamp GWH (1992). Stromal expression of 72 kDa type IV collagenase (MMP-2) and TIMP-2 mRNAs in colorectal neoplasia. *American Journal of Pathology* **141**(2) 389-395.

Stetler-Stevenson WG (1996). Dynamics of matrix turnover during pathologic remodeling of the extracellular matrix**.** *American Journal of Pathology* **148**(5) 1345-1350.

Towbin H, Staehelin T and Gordon J (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitro cellulose sheets: procedure and some applications. *Proceedings of National Academic Sciences(USA)***76**(9) 4350-4355.