



***In-vitro* study of matrix metalloproteinases in decellularized extracellular matrix of bovine diaphragm**

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ABSTRACT

The present study was undertaken for the identification of matrix metalloproteinases (MMPs) in the extracts obtained from a multi-step detergent-enzymatic extraction process involving 2 types of anionic and non-ionic detergents from bovine diaphragm and their possible involvement in the degradation of these biological materials. Anionic biological detergents treated bovine diaphragm expressed no band in SDS-PAGE analysis but non-ionic treated bovine diaphragm expressed almost similar bands as that of untreated treated bovine diaphragm. In gelatin zymography, anionic treated biomaterial expressed a 25 kDa band of MMPs, but in non-treated and non-ionic treated bovine diaphragm no band of MMPs was expressed. Anionic detergents played an important role in the removal of cells from bovine diaphragm and increasing MMPs activity in biomaterials as compared to non-ionic detergents.

Key words: Bovine diaphragm, Gelatin zymography, MMPs, SDS-PAGE electrophoresis

A class of enzymes known as matrix metalloproteinases (MMPs) have role in connective tissue degradation and remodelling. The MMPs are a family of endopeptidases which digest proteins of the extracellular matrix (ECM). The MMPs were grouped into 3 subfamilies—collagenase, gelatinase, and stromelysin—based on the specificity for different extracellular matrix components (Woessner *et al.* 1991). The interstitial collagenase, designated as MMP-1, degrades native collagen types I, II, and III. Stromelysin (MMP-3) cleaves proteoglycans, fibronectin, laminin, gelatins and casein. The 72-kDa gelatinase (MMP-2) and the 92-kDa gelatinase (MMP-9) showed activities against gelatins of all types and native collagen types IV, V, and VII. The MMPs family members share considerable sequence similarity and the different proteinases appear to have evolved from a common ancestral gene (Fini *et al.* 1987). These enzymes are secreted as proenzymes and are activated by cleavage of a polypeptide from the amino terminal portion of the molecule. They are important for regulating normal tissue development in morphogenesis, angiogenesis and wound healing (Matsubara *et al.* 1991). Abnormal expression or unregulated activities of MMPs was associated with pathologic conditions including rheumatoid arthritis,

atherosclerosis, cancer expansion, invasion and metastasis (Liotta *et al.* 1986). Concerning their possible involvement in biomaterial degradation, Simionescu *et al.* (1996 a,b) reported the presence of MMPs in diseased human valves and in bioprostheses made of glutaraldehyde pretreated bovine pericardium.

The aim of this work was to analyze the presence of MMPs activity in bovine diaphragm and their possible involvement in the degradation of these biological materials. The tissue was made acellular and the decellularization techniques include chemical, enzymatic and mechanical means of removing cellular components, leaving a material composed essentially of extracellular matrix components.

MATERIALS AND METHODS

Experimental design

Fresh bovine diaphragm was procured from the local abattoir. Immediately after the collection the biomaterial was preserved in cold physiological saline solution. The tissue was gently rinsed with fresh saline to remove the adhered blood. Biomaterials were subjected to different protocols to make completely acellular tissue matrices by specifically removing cellular components. The protocols included use of different chemicals detergents and enzymes for removing cellular components, leaving a material composed essentially of extra cellular matrix (ECM) components. Two anionic and 2 non-ionic detergents were used for making the tissue acellular.

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Protocol I– Using enzyme + anionic detergents: Two different anionic biological detergents SDO and SDS were used in 0.5% concentration. The samples were continuously agitated with 0.5% of trypsin for 24 h along with protease inhibitors. Later on, the trypsin was replaced with 0.5% solution of anionic biological detergents (SDO and SDS) and agitated for next 72 h. Final washing was done with phosphate buffer and stored at -20°C .

Protocol II– Using Enzyme + non-ionic detergents: Two different non-ionic biological detergents TX and TW were used in 0.5% concentration. The samples were continuously agitated with 0.5% of trypsin for 24 h along with protease inhibitors. Later on, the trypsin was replaced with 0.5% solution of non-ionic biological detergents (TX and TW) and agitated for next 72 h. Final washing was done with phosphate buffer and stored at -20°C .

Sample preparation: Native and acellular tissue samples were washed 3 times with phosphate buffer saline (pH 7.4). Each tissue of 1 g was weighed out, minced with scissors and homogenized with 5 ml of homogenizer tissue mixture (50 mM Tris pH 8.0, 0.9% NaCl, 0.25% Triton X100) in homogenizer. The homogenate was centrifuged at 3000 rpm for 10 min. The supernatant was separated and stored at -20°C for further use.

SDS-PAGE electrophoresis: SDS-PAGE electrophoresis was performed using 10% total acrylamide under non-reducing condition as per method described by Laemmli (1970).

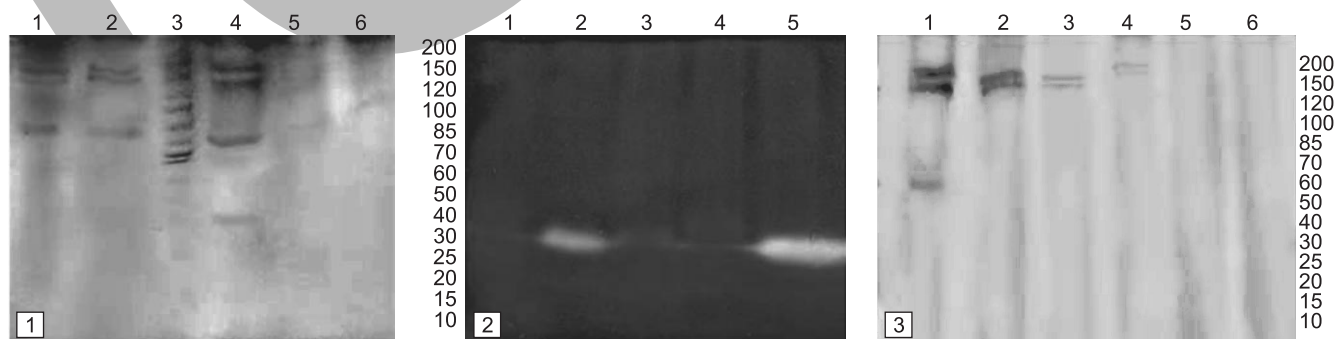
Zymography: Zymography was performed as per Huessen and Dowelle (1980) in sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis with gels containing substrates, such as gelatin (for gelatinase) to analyze the content of specific proteinases in the homogenized tissue samples of bovine diaphragm. In brief, 10% SDS-Polyacrylamide gels were prepared and 0.5% of gelatin (type A) was co-polymerized in the gel. Tissue extracts samples (25 μl each) were mixed with an equal volume of 5X sample buffer, loaded onto the gel without boiling and electrophoresis

was done under non reducing conditions. The gel was then shaken for 1 h in a 2.5% solution of Triton X-100 at room temperature to remove the SDS and incubated at 37°C in a reaction buffer (50 mM Tris, pH 7.5; 10 mM CaCl_2) overnight to allow proteinase digestion of the substrates. After staining with Coomassie brilliant blue R-250, the positions of enzymatic activities were identified as clear bands in the stained background.

Effect of enzymes on MMPs and protein concentration: Bovine diaphragm homogenate was incubated with equal volume of different enzymes pepsin and papain (1% in distilled water), trypsin, collagenase and elastase (1% in phosphate buffer saline) for the period of 16 h at 37°C . SDS-PAGE and gelatin zymography was performed after incubation of samples with different enzymes. The final concentration of each sample was 30 μl / lane.

RESULTS AND DISCUSSION

Electrophoretic analysis: The electrophoretic analysis of equivalent volumes of the supernatants shows the different efficiencies of the homogenization process. Native diaphragm showed strongly stained bands at 150–160 KDa and fainter low molecular weight bands at 45 and 85 kDa in lane 4. On the other hand, no bands were observed in tissue samples treated with anionic detergents in lane 5 and 6 (Fig.1), which indicates the removal of cytoplasmic cellular components, lipids and its membranes along with soluble proteins by decellularization techniques. Decellularization of biomaterials make the tissue less antigenic, reducing the inflammatory response and causing less tissue degeneration (Elkins *et al.* 2001, Grauss *et al.* 2003). Courtman *et al.* (1994) reported that acellular biological tissues have been used as natural biomaterials for tissue repair. Tissue treated with non-ionic biological detergents expressed almost similar protein bands as compared to native diaphragm. These results showed that anionic detergents were better detergents as compared to non-ionic biological detergents for the removal of the cells from the bovine diaphragm.



Figs 1–3. **1.** SDS Page of bovine diaphragm. Lane 1, non-ionic TX treated; lane 2, non-ionic TW treated; lane 3, marker; lane 4, native tissue; lane 5, anionic SDS treated, lane 6, anionic SDS treated **2.** Gelatin Zymography of bovine diaphragm; lane 1, native tissue; lane 2, anionic SDS treated; lane 3, non-ionic TX treated; lane 4, non-ionic TW treated; lane 5, anionic SDO treated. **3.** SDS Page of bovine diaphragm treated with different enzymes. Lane 1, native tissue; lane 2, pepsin treated; lane 3, papain treated; lane 4, trypsin treated; lane 5, collagenase treated; lane 6, elastase treated.

No protein bands were observed in lane 5 and 6 in Fig. 3, which were treated with collagenase and elastase but the expression of protein bands were similar in papain and pepsin enzymes treated samples as shown in lane 2 and 3. In lane 4, a very faint band around 150 and 160 KDa was expressed, which was treated with trypsin enzyme. We observed that bovine diaphragm showed highest enzymatic degradation with collagenase and elastase enzymes followed by trypsin, papain and pepsin. Similar findings were reported by Liang *et al.* (2004) and Vishu Kumar *et al.* (2005).

Gelatin zymography: Various techniques are used to determine the presence of proteolytic enzymes in tissues. Zymography is a simple, sensitive, quantifiable, and functional approach for the analysis of proteolytic activity in cells and tissue extracts. During the past decade, *in situ* zymography was applied to localize gelatinase activity in tissue sections (Galis *et al.* 1994). Gelatin zymography was used to detect gelatinase activities in the supernatants from the homogenisation of the different chemically modified tissue samples of bovine diaphragm. Snoek van Beurden and Von den Hoff (2005) reported that zymography identifies MMPs by the degradation of their preferential substrate and by their molecular weight.

In the lane 1 corresponding to the untreated diaphragm (Fig.2) and lane 3, 4 treated with non-ionic detergents showed, a very faint band of MMPs around 25 KDa but anionic detergents treated tissue showed strong band of around 25 KDa. The relative amount of the 25 KDa was 1–2 times higher in SDO treated lane as compared to SDS treated lane. The extraction of cellular components from tissues minimized immunologically induced inflammatory processes. Wilson *et al.* (1990) reported that acellular matrix vascular allografts of canine arteries that were produced by detergent and enzymatic extraction showed no inflammation when implanted into dogs of a different breed. Bergmann *et al.* (1989) reported that macrophages and granulocytes are not believed to function in blood but it is possible that these cells secrete the protease during their invasion and phagocytic action in tissues which may leak into plasma where it could be measured by ELISA technique. MMPs activity was completely inhibited by collagenase, elastase, trypsin, pepsin and papain enzymes in bovine diaphragm homogenates. These results indicated that anionic detergents participate for the increasing MMPs activity in bovine diaphragm as compared to non-ionic detergents.

REFERENCES

- Bergmann U, Michaelis J, Oberhoff R, Knqer V, Beckmann R and Tschesche H. 1989. Kind of sample as preanalytical determinant of matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinase 2 in blood. *Journal of Clinical Chemistry and Clinical Biochemistry* **27**: 351–59.
- Courtman D W, Pereira C A, Kashef V, McComb D, Lee J M and Wilson G J. 1994. Development of a pericardial acellular matrix biomaterial biochemical and mechanical effects of cell extraction. *Journal of Biomedical Material Research* **28**: 655–66.
- Elkins R C, Dawson P E, Goldstein S, Walsh S P, Black K S. 2001. Decellularized human valve allografts. *Annals of Thoracic Surgery* **71**: S28–32.
- Finini M E, Plucinska I M, Mayer A S, Gross R H, Brinckerhoff C E. 1987. A gene for rabbit synovial cell collagenase: Member of a family of metalloproteinases which degrades the connective tissue matrix. *Biochemistry* **26**: 6156–64.
- Galis Z S M, Muszynski M, Sikhova G K, Simon-Morrissey E, Unemori E N, Lark, M W, Amento E and Libby P. 1994. Cytokine-stimulated human vascular smooth muscle cells synthesize a complement of enzymes required for extracellular matrix digestion. *Circulation Research* **75**:181–89.
- Grauss R W, Hazekamp M G, Vleit S, Gittenberger-de-Groot A C, DeRuiter M C. 2003. Decellularization of rat aortic valve allografts reduces leaflet destruction and extracellular matrix remodeling. *Journal of Thoracic and Cardiovascular Surgery* **126**: 5–10.
- Huessen C and Dowdle E B. 1980. Electrophoretic analysis of plasminogen activator in polyacrylamide gels containing sodium dodecylsulfate and copolymerized substrates. *Annals of Biochemistry* **102**: 96–102.
- Laemmli U K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–85.
- Liang H C, Chang Y, Hsu C K, Lee M H and Sung H W. 2004. Effect of cross-linking degree on an acellular biological tissue on its tissue regeneration pattern. *Biomaterials* **25**: 3541–52.
- Matsubara M, Girard M T, Kublin C L, Cintron C, Fini M E. 1991. Differential roles for two gelatinolytic enzymes of the matrix metalloproteinase family in the remodelling cornea. *Developmental Biology* **147**: 425–39.
- Simionescu A, Simionescu D and Deac R F.1996. Biochemical pathways of tissue degeneration in bioprosthetic cardiac valves. *ASAIO Journal* **42**: M561–70.
- Simionescu A, Simionescu D and Deac R F.1996. Matrix metalloproteinases in the pathology of natural and bioprosthetic cardiac valves. *Cardiovascular Pathology* **5**: 323–32.
- Snoek van Beurden P A M and Von den Hoff J W. 2005. Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. *BioTechniques* **38**: 73–83.
- Vishu Kumar A B, Varadaraj M C, Goeda L R, Tharanathan R N. 2005. Characterization of chito-oligosaccharides prepared by chitosan analysis with the aid of papain and pronase and their bacterial action against *Bacillus cereus* and *Escherichia coli*. *Biochemistry* **391**: 167–75.
- Woessner J F Jr. 1991. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB Journal* **5**: 2145–54.