

# Thermolysin Activates Equine Lamellar Hoof Matrix Metalloproteinases

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## Summary

Cultured equine lamellar hoof explants secrete the pro-enzymes matrix metalloproteinase-2 (MMP-2, 72 kDa) and MMP-9 (92 kDa). Untreated explants remained intact when tested on a calibrated force transducer, but when treated with an MMP activator, developed "in-vitro laminitis", separating at the dermal-epidermal junction. Explants treated with the bacterial protease thermolysin separated dose-dependently; this was accompanied by activation of both MMP-2 and -9. Thermolysin-mediated MMP activation did not occur in a cell-free system and was not inhibited by the addition of the MMP inhibitor and batimastat. These findings suggest that thermolysin-mediated gelatinase activation is not dependent on membrane-bound matrix metalloproteinase (MT-MMP) activation, providing further evidence that bacteria can produce potent MMP activators that probably facilitate host invasion.

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## Introduction

Matrix metalloproteinases (MMPs) are a family of zinc-containing, calcium-dependent, proteolytic enzymes that play a role in normal physiological remodelling of the extracellular matrix (Birkedal-Hansen, 1993). With the exception of membrane-bound MMPs (MT-MMPs), all these enzymes are secreted as inactive pro-enzymes, which require limited proteolysis to attain activation. MMP activation is believed to occur either via proteolytic cleavage of the pro-peptide by other proteinases or autocatalytically (Kleiner and Stetler-Stevenson, 1993). *In vitro*, all the MMPs can be chemically activated by organomercurial compounds such as p-aminophenyl-mercuric acetate (APMA) (Okada *et al.*, 1990); activation *in vivo* has been identified only recently (Okamoto *et al.*, 1997a). While MMP-2 appears to be the only true autocatalytic MMP, several MMPs are capable of activating other members of the family (He *et al.*, 1989; Suzuki *et al.*, 1990; Knauper *et al.*, 1993; Crabbe *et al.*, 1994; Sato *et al.*, 1994; Sang *et al.*, 1995). In addition,

some MMPs are activated by plasmin (Collier and Ghosh, 1988; Gavrilovic and Murphy, 1989; Campbell *et al.*, 1990; Murphy *et al.*, 1992) trypsin, chymotrypsin, cathepsin-G (Grant *et al.*, 1992; Morodomi *et al.*, 1992) and proteinases from mast cells (Suzuki *et al.*, 1995).

Many physiological compounds, most notably cytokines and other growth factors (Mackay *et al.*, 1992; Mann *et al.*, 1995; Makela *et al.*, 1998; Zhang *et al.*, 1998) have been implicated as MMP "modulators", with a role in regulating the levels of MMP transcription and production; to date, however, these have not been linked directly to MMP activation. Endogenous proteinases are likely to be responsible for MMP activation under most physiological and patho-physiological situations, but the possibility of exogenous proteinase-mediated activation has been explored only recently (Okamoto *et al.*, 1997b). Sorsa *et al.* (1992) identified proteases from periodontopathogenic bacteria capable of activating latent human fibroblast type and neutrophil interstitial procollagenases, and Matsumoto *et al.* (1992) showed that *Pseudomonas aeruginosa* elastase was capable of activating MMP-2.

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Subsequent research by Okamoto *et al.* (1997b) revealed that MMP-1, -8 and -9 could all be activated by bacterial proteinases belonging to the thermolysin family (M4) (Rawlings and Barrett, 1995). Specifically, *Ps. aeruginosa* elastase, *Vibrio cholerae* protease and thermolysin showed strong activation of all three pro-MMPs.

MMP-2 and -9 are present in equine lamellar hoof and the key lesion of equine laminitis (separation of epidermis from dermis at the dermo-epidermal junction) may be due to inappropriate activation of lamellar MMPs. Basement membranes are target substrates for MMP activity and lysis, and separation of the lamellar basement membrane is present soon after the development of acute laminitis (Pollitt, 1996; Pollitt and Daradka, 1998). Chemical activation of hoof lamellar MMPs by the addition of APMA produces a rapid and reproducible in-vitro lamellar separation readily inhibited by the MMP inhibitor batimastat (Pollitt *et al.*, 1998).

This paper describes a study showing that thermolysin activates MMP-2 and -9 in equine hoof explants and results in a laminitis-like separation between the dermal and epidermal lamellae, apparently via a cell-mediated process independent of MT-MMP.

## Materials and Methods

### *Explant Culture and Assessment of Structural Integrity*

Hooves from 10 normal, disease-free horses (inspected by a veterinarian) were obtained from a commercial abattoir exporting meat for human consumption. Hooves were selected at random and only those with no sign of foot disease (acute or chronic laminitis) or abnormal growth were used. Data on age, gender or performance history were not available. Hooves were transported on ice to the dissection room within 60 min of slaughter. They were then dissected to obtain pieces of tissue ( $c. 6 \text{ mm}^3$ ) extending from the inner hoof wall through the lamellar junction to the dermal connective tissue (Pollitt *et al.*, 1998). The tissue samples were incubated in Dulbecco's Modified Eagles Medium (D-MEM, BioWhittaker, Maryland, USA) containing either thermolysin (Sigma, St Louis, USA) (100 ng/ml–100  $\mu\text{M}$ /ml), thermolysin and batimastat (British Biotechnology, Oxford, UK) (50  $\mu\text{M}$ –100  $\mu\text{M}$ ) or 1 mM ethylene-diamine-tetraacetic acid (EDTA; Sigma) at 37°C, in an atmosphere of 95% relative humidity containing CO<sub>2</sub> 5% for 48 h. Supernates were collected and stored at –80°C until assayed.

Explants were examined for structural integrity by strain gauge assessment. One end of the explant was immobilized by attachment to a fixed alligator clip, and the other was attached to a calibrated force transducer, also via alligator clip. Tissues were stretched up to a maximum force of 700 g. The force required to separate the explant at the dermal epidermal junction was recorded. Tissues remaining "intact" were given the maximum score of 700 g. Separation forces were converted to a "% separation" score ( $[700 - \text{force required}] \div 700 \times 100$ ), where 0 g = 100% separation and 700 g = 0% separation. Scores were averaged over five experiments (five different horses, cultured in duplicate) and statistical analysis was performed with a repeated measure analysis of variance procedure (Dunnett's test). Aliquots of all media were stored at –80°C until assessed by SDS-PAGE zymography.

### *In-vitro Tube Assay*

Cell-free MMP activation was examined in spent explant medium treated with thermolysin (1 ng/ml–10  $\mu\text{g}$ /ml), thermolysin in the presence of batimastat (100  $\mu\text{M}$ ) or APMA (0.25 mg/ml), in a tube assay system (20  $\mu\text{l}$  medium, 16  $\mu\text{l}$  treatment compound, 8  $\mu\text{l}$  incubation buffer [50 mM Tris-HCl, 5 mM calcium chloride and 0.02% sodium azide], final reaction volume 40  $\mu\text{l}$ ). Components were added, "vortexed" briefly, pulse centrifuged and incubated at 37°C for 15, 30, 60 or 120 min. Samples were immediately frozen at –80°C until assessed by SDS-PAGE zymography.

### *SDS-PAGE Zymography*

Samples were subjected to electrophoresis for 60 min (30 mA, 10°C) on non-reducing 7.5–15% gradient polyacrylamide gels containing gelatin 0.1% under non-reducing conditions, as described previously (Pollitt *et al.*, 1998). The gels were next washed in 2.5% Triton X-100 (Sigma) for 2  $\times$  30 min, and incubated for 24 h at 37°C in incubation buffer. They were then stained with Coomassie blue G-250 (30 min), destained with acetic acid 5% and glycerol 2% in water (24 h), dried with a Biorad gel dryer and scanned with an HP Scanjet 3C flatbed scanner (Hewlett Packard) and densitometry analysis software (GelPro, Media Cybernetics, Silver Springs, USA). Briefly, integrated optical density (IOD) measurements were produced by calculating the area under the curve of all bands in a graphical representation of optical density measurements derived from scanning zymography gels. IOD measurements were averaged

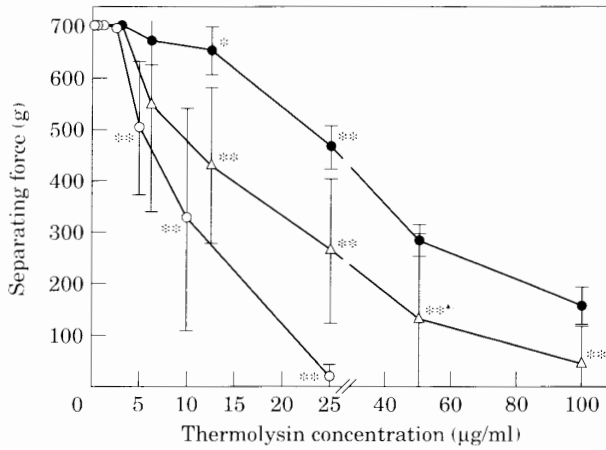


Fig. 1. Effect of thermolysin in the force (mean  $\pm$  SEM) required to separate explants alone or in the presence of batimastat or EDTA during culture for 48 h ( $n=5$ ). Thermolysin  $\circ$ , thermolysin + batimastat (50 mM)  $\triangle$ , thermolysin + batimastat (100 mM)  $\square$ ,  $\bullet$ ; \* $P<0.05$ , \*\* $P<0.01$ .

from five experiments and significance was calculated by means of a repeated measures analysis of variance procedure (Dunnett's test).

## Results

### *In-vitro* Explant Assay

Equine hoof explants cultured in medium alone did not separate ( $n=10$ ); explants treated with thermolysin, however, separated in a dose-dependent manner (Fig. 1). While there was a trend towards separation at thermolysin 2.5  $\mu\text{g/ml}$ , this was not statistically significant. Thermolysin 5  $\mu\text{g/ml}$  reproducibly caused explant separation ( $P<0.01$ ,  $n=5$ ), although considerable force was required. Explants separated more easily as the concentration of thermolysin increased; thus, only 50% of the maximum force was required after incubation with thermolysin 10  $\mu\text{g/ml}$  and almost no force after incubation with thermolysin 26  $\mu\text{g/ml}$  (Fig. 1).

Thermolysin-mediated separation was inhibited in a dose-dependent manner by the addition of batimastat to the culture medium (Fig. 1). Addition of 50  $\mu\text{M}$  batimastat to explants treated with thermolysin resulted in a significant increase in the force required to separate explants (Fig. 1). With 100  $\mu\text{M}$  batimastat (Fig. 1), thermolysin could still separate explants, but a 10-fold increase in concentration was required (Fig. 1). The general MMP inhibitor, EDTA (1 mM), also inhibited thermolysin-mediated separation, the inhibition being equivalent to that produced by 50  $\mu\text{M}$  batimastat (Fig. 1).

### SDS-PAGE Zymography

The results obtained with explant medium showed activation of pro-MMP-2 in a dose-dependent manner (Fig. 2) and the degradation of pro-MMP-9. As little thermolysin as 100 ng/ml activated pro-MMP-2 (72 kDa) produced by explants to its 66 kDa active form, with progressive activation to a smaller 62 kDa active form occurring with thermolysin concentrations of  $\geq 312.5$  ng/ml (Fig. 2). Pro-MMP-9 (92 kDa) produced by explants was converted into its 82 kDa active form in the presence of thermolysin (Fig. 2); this was, however, progressively degraded by increased concentrations of thermolysin. Addition of batimastat or EDTA had no effect on the efficacy of MMP-2 or MMP-9 activation (data not shown).

The relative quantities of the pro (72 kDa) and active isoforms of MMP-2 (66 and 62 kDa) followed an inverse relationship (Fig. 3a), while MMP-9 (92 and 82 kDa, pro and active forms, respectively) appeared to be degraded less specifically (Fig. 3b). Reflecting the conversion of pro-MMP-2 (72 kDa) to its smaller active form, thermolysin reduced the quantity of 72 kDa MMP-2 while proportionately increasing the quantity of 66 kDa MMP-2 (Fig. 3a). Slightly higher doses of thermolysin further converted the excess 66 kDa form to a smaller 62 kDa form, essentially halting the rise in 66 kDa concentration (Fig. 3a). Thermolysin alone was observed to exhibit gelatinase activity at concentrations of  $\geq 1$   $\mu\text{g/ml}$  (Fig. 4).

### *In-vitro* Tube Assay

Media collected from untreated explants and incubated with thermolysin exhibited no activation of either MMP-2 or -9 at any concentration (0.01–10  $\mu\text{g/ml}$ ) throughout the range of incubation times examined (15–120 min). Media treated with APMA exhibited partial activation of pro-MMP-2 (72 kDa) and almost complete activation of pro-MMP-9 (92 kDa) after incubation for 15 min, with  $>50\%$  of 72 kDa MMP-2 converted to its 66 kDa form after incubation for 2 h (Fig. 4). Batimastat had no effect on thermolysin- or APMA-mediated activation of either MMP-2 or -9 (data not shown).

## Discussion

The present study highlights the pathogenic potential of bacterial proteases in MMP processing and activation. MMPs are a family of enzymes capable of breaking down proteins of the extracellular matrix. They have been implicated in many

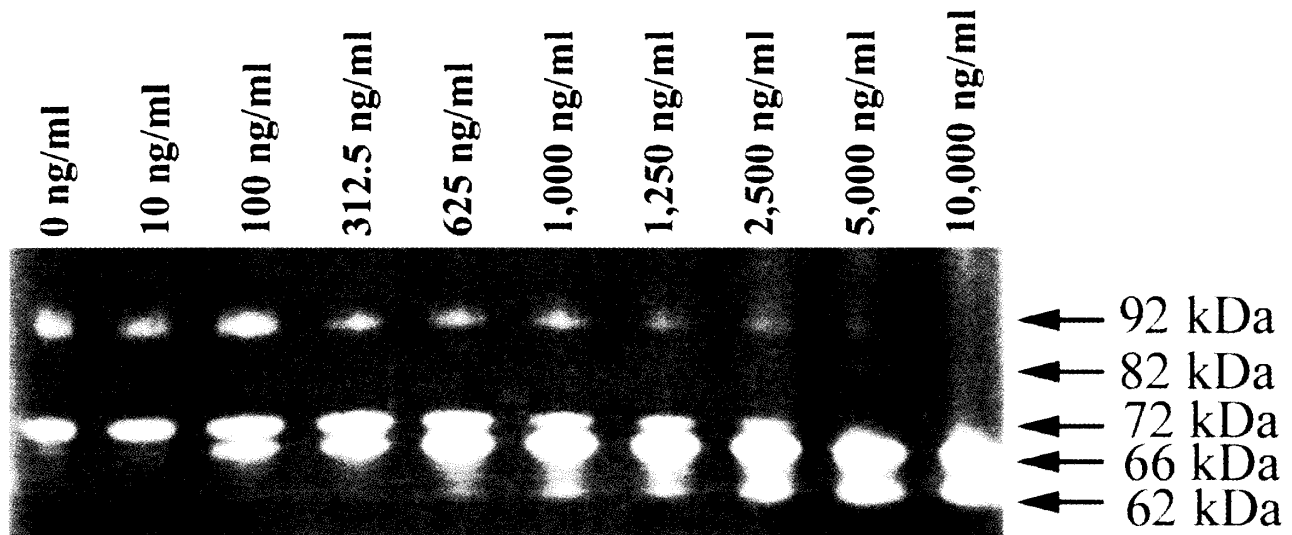


Fig. 2. Typical SDS-PAGE zymography of explant medium cultured with increasing concentrations of thermolysin, showing progressive activation of MMP-9 (92 and 82 kDa) and MMP-2 (72, 66 and 62 kDa). Arrows indicate molecular weights (MWs).

physiological processes including normal tissue growth and remodelling (Matrisian, 1992; Sato *et al.*, 1994; Birkedal-Hansen, 1995; Kahari and Saarialho-Kere, 1997) and trophoblast invasion and placentation (Huppertz *et al.*, 1997; Rudolph-Owen *et al.*, 1997). From a clinical perspective, their roles in cancer metastasis (Stetler-Stevenson, 1990) and bullous skin diseases (see Kahari and Saarialho-Kere, 1997) have been the focus of considerable research. Central to much of this research is the mechanism of MMP activation. In an *in-vitro* model of equine laminitis, hoof lamellar explants cultured in D-MEM secrete the gelatinases pro-MMP-2 (72 kDa) and pro-MMP-9 (92 kDa) (Pollitt *et al.*, 1998). In the absence of gelatinase activation, these explants cannot be separated into their dermal and epidermal compartments by the application of a stretching force, i.e., they remain "intact". Chemical activation of these gelatinases by the addition of APMA produced a rapid and reproducible lamellar separation readily inhibited by the MMP inhibitor batimastat in studies by Pollitt *et al.* (1998), who postulated that the key lesion of equine laminitis is a direct result of inappropriate MMP regulation within the lamellar structure. Despite extensive testing, the search for a physiological MMP activator in the *in-vitro* laminitis model has been fruitless. While the modulation of MMPs by cytokines and other growth factors is well established in many tissues and cell lines (Mackay *et al.*, 1992; Mann *et al.*, 1995; Makela *et al.*, 1998; Zhang *et al.*, 1998), the addition of

recombinant cytokines to equine hoof explant cultures produced only moderate effects on gelatinase production and failed to influence MMP activation (Mungall and Pollitt, unpublished data).

Addition of thermolysin to lamellar explant cultures readily produced a dose-dependent separation of the dermis from the epidermis (*in-vitro* laminitis) accompanied by a dose-dependent activation of pro-MMP-2 and pro-MMP-9. While thermolysin had its own gelatinolytic activity (Fig. 4), MMP activation was observed at concentrations lower than that producing gelatinolytic activity detectable by conventional SDS-PAGE zymography. Separation, but not activation, was significantly inhibited by the addition of a specific MMP inhibitor, batimastat, and by the addition of EDTA, indicating the role of MMP activation in the lamellar separation process. Furthermore, the lack of thermolysin-mediated MMP activation in a cell-free system (Fig. 4) indicated that thermolysin was probably acting via some cell-mediated process.

*In vitro*, and presumably *in vivo*, pro-MMP-9 can be activated by several different mechanisms including limited proteolysis by trypsin, plasmin, cathepsin G and chymotrypsin (Grant *et al.*, 1992; Morodomi *et al.*, 1992), while pro-MMP-2 is surprisingly resistant to proteolytic activation (Okada *et al.*, 1990; Woessner, 1991). MMP-2 can be reproducibly activated in cell culture by several exogenous compounds, including phorbol 12-myristate 13-acetate (PMA) (Foda *et al.*, 1996), concanavalin A (Overall and Sodek, 1990), cytochalasin D (Allen-

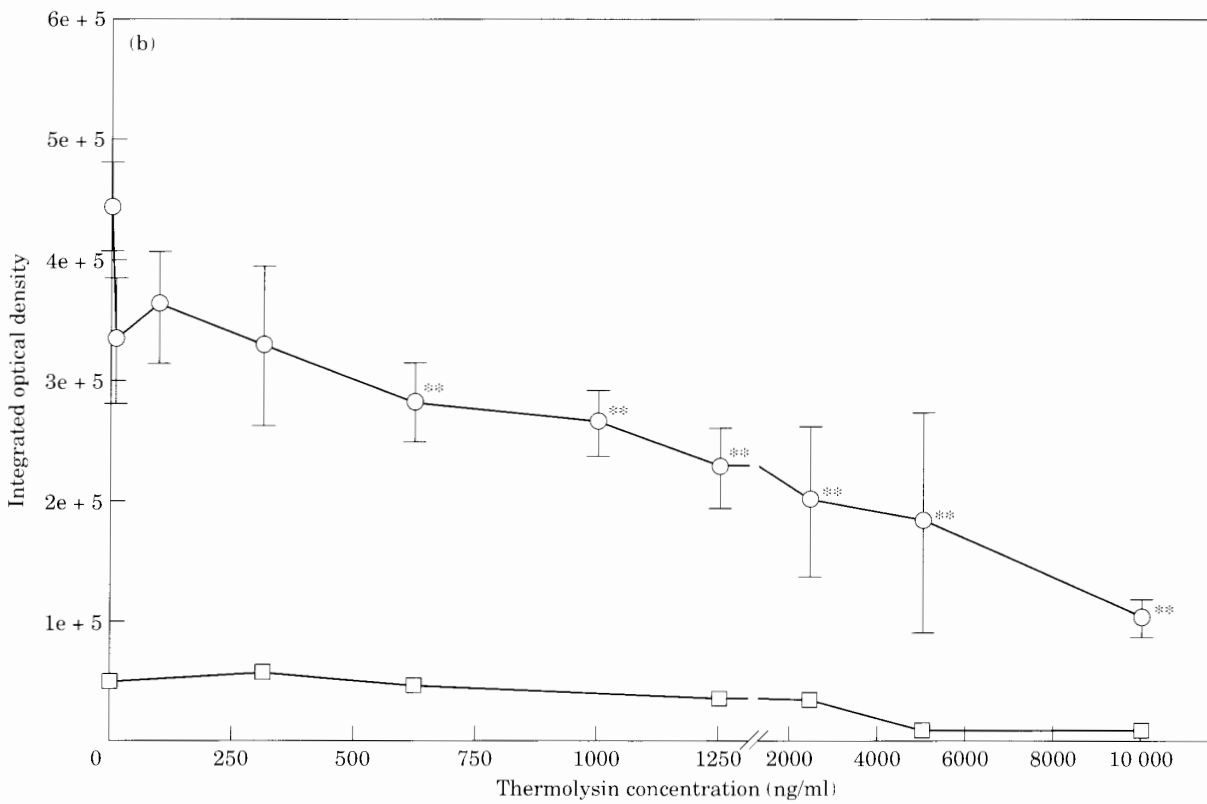
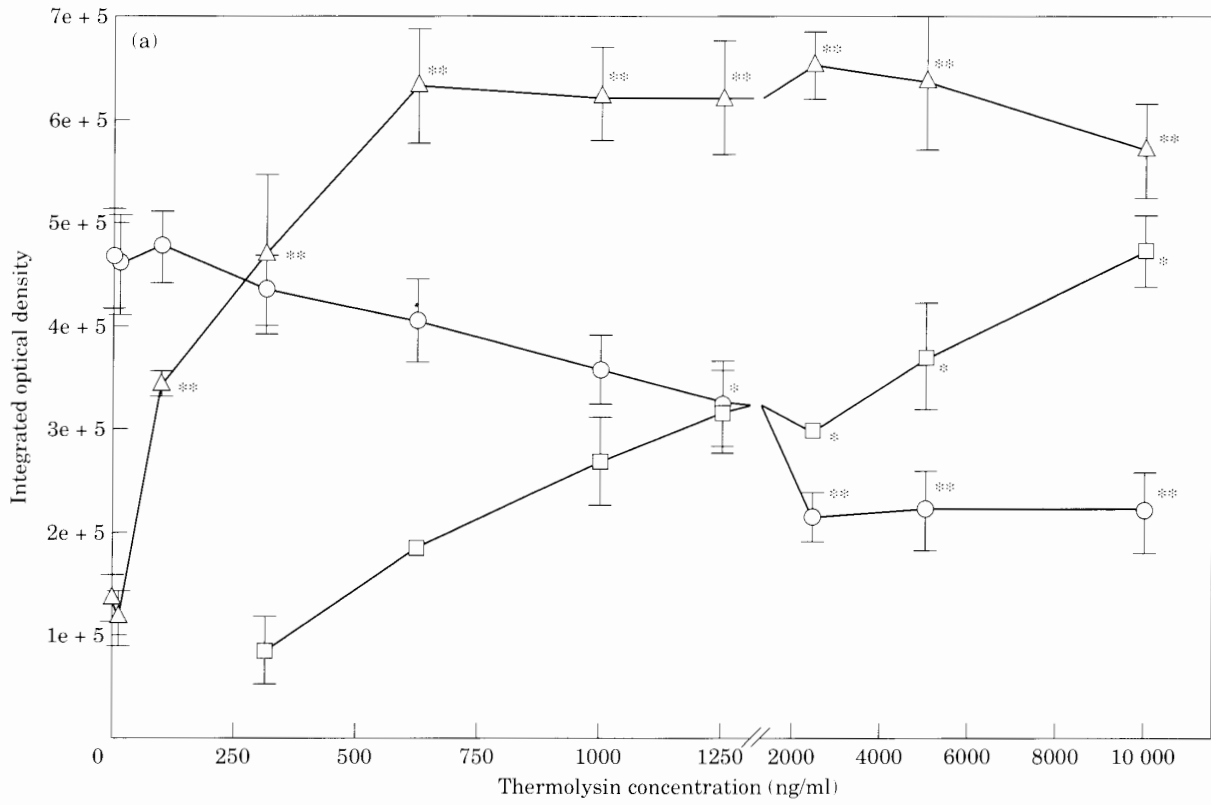


Fig. 3a,b. Integrated optical density (mean  $\pm$  SEM) of SDS-PAGE zymography bands corresponding to (a) MMP-2 isotypes (72 kD  $\circ$ , 66 kD  $\triangle$ , 62 kD  $\square$ ; \* $p$ <0.05, \*\* $p$ <0.01), and (b) MMP-9 isotypes present in explant media conditioned by increasing concentrations of thermolysin during culture for 48 h ( $n=5$ ) (92 kD  $\circ$ , 82 kD  $\square$ ; \*\* $p$ <0.01).

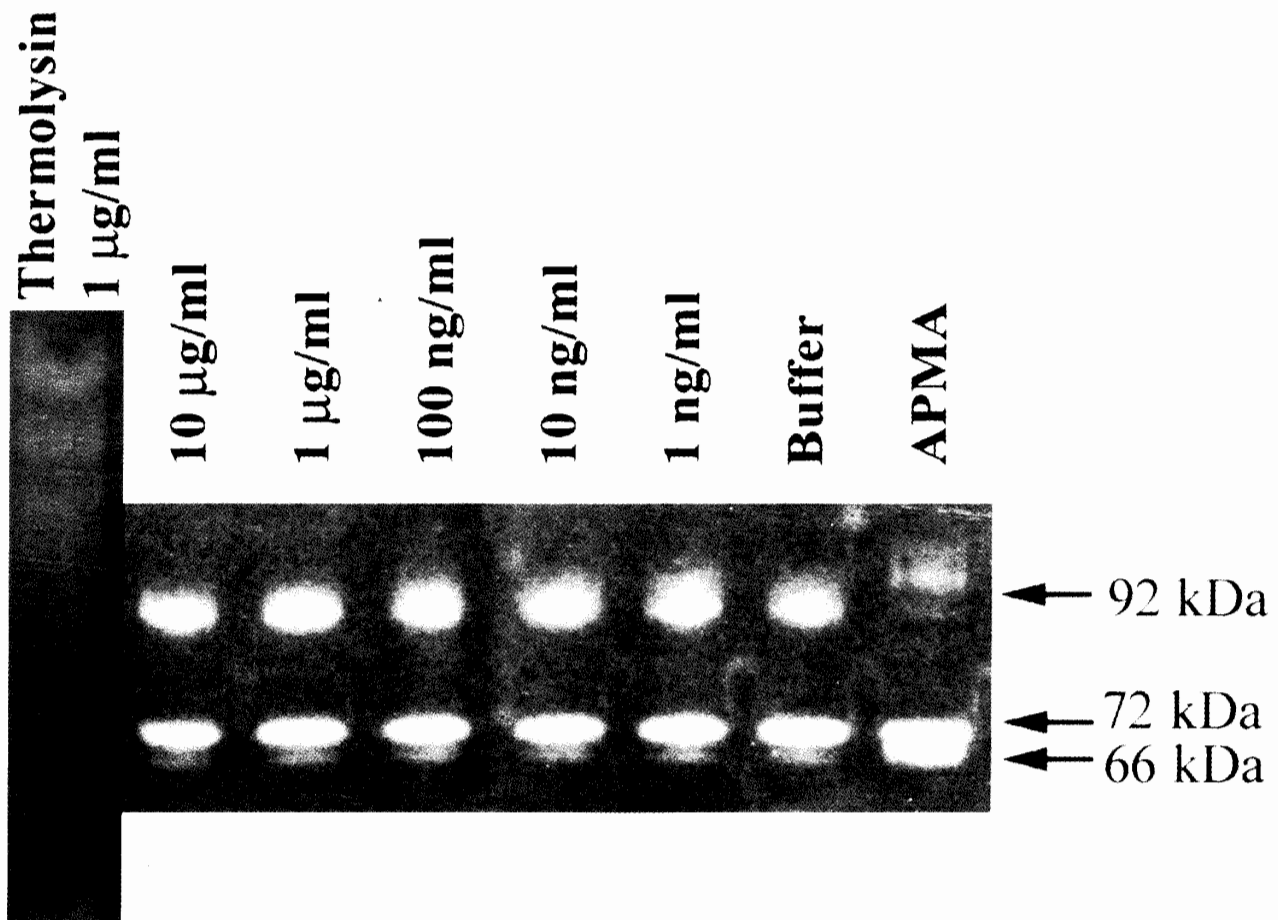


Fig. 1. Typical SDS-PAGE zymography of spent explant medium incubated with increasing concentrations of thermolysin, showing no visible signs of MMP activation. Incubation with APMA resulted in partial activation of MMP-2, converting its 72 kDa isotype to a smaller 66 kDa isotype, in addition to partial degradation of MMP-9 (92 kDa), often without visible production of a smaller corresponding fragment. For comparison, zymography of high concentrations of pure thermolysin usually resulted in several bands of a much larger apparent MW (corresponding to dimers, tetramers etc.).

berg *et al.*, 1994; Ailenberg and Silverman, 1996) and monensin (Li *et al.*, 1997), but in-vivo activation is still poorly understood. Although second messenger systems are being implicated in MMP-2 activation (Foda *et al.*, 1996; Li *et al.*, 1998; Yu *et al.*, 1998), all of these "activators" also elevate the levels of MT-MMP (Yu *et al.*, 1995; Ailenberg and Silverman, 1996; Foda *et al.*, 1996; Lohi *et al.*, 1996; Li *et al.*, 1997). Current theories suggest that the primary role of MT-MMPs is related to MMP-2 activation (Peracchia *et al.*, 1997; Murphy *et al.*, 1999; Seiki, 1999). However, thrombin has recently been shown to activate MMP-2 via an MT-MMP-independent process in microvascular epithelial cells (Nguyen *et al.*, 1999).

The observation in this study that MMP activation by thermolysin was not inhibited by batimastat indicates two possibilities, namely (1) that

thermolysin is not a conventional MMP and therefore resists such inhibition, and (2) that MMP-2 activation probably occurs independently of MT-MMP activation, the latter being susceptible to MMP inhibitors (Nguyen *et al.*, 1999). Additionally, thermolysin was unable to generate active MMP-2 and -9 in a cell free system, indicating a requirement for cell or tissue contact. Taken together, these observations suggest that thermolysin acts in concert with other proteins *in vivo* as an exogenous MMP activator.

This study showed that a bacterial product was capable of tissue-dependent activation of MMP-2 and -9, apparently independently of MT-MMP. The possibilities that thermolysin is a MT-MMP-like activator of MMP-2 and -9, and that bacteria have evolved complex pathogenic strategies based on utilization of the host's natural proteo-

lytic enzyme systems, require further investigation.

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