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Zymography-Based Assay for Screening Potential Anti-Gelatinase Agents using *Serratia marcescens* Serralysin

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Matrix metalloproteinases (MMPs) are a class of over 20 zinc-dependent proteolytic enzymes that have been implicated in the physiologic process of aging and in the pathogenesis of cancer. Specifically, the gelatinases MMP-2 and MMP-9 have been intensively studied in connection with tumor invasion and metastasis, and have been considered as promising targets for cancer treatment. Herein, we introduce a fast, reliable and cost-effective zymography-based assay that can be used routinely for pre-screening agents with potential anti-gelatinolytic activity. Agents which exhibit anti-gelatinolytic activity in this assay can then be tested using conventional screening assays for anticancer agents. The gelatinase serralysin from culture supernatants of the Gram-negative bacterium *Serratia marcescens* was employed. Several anti-aging creams and plant extracts were tested. Vitamin E- and tretinoin-containing creams showed remarkable anti-gelatinolytic activity. For the natural products, aqueous extract of papaya gave the most remarkable anti-gelatinolytic activity as compared to aqueous extracts of guava and tomato. To verify these results, culture supernatants of normal human keratinocytes and HaCaT cells were treated with the same agents. Keratinocyte-secreted gelatinases (MMP-2, MMP-9) and *Serratia marcescens*–secreted gelatinase (serralysin) were inhibited in a similar manner by the tested anti-aging creams and by papaya extract. Taken together, zymography using gelatinases from microorganisms can serve as a useful tool for pre-screening synthetic and natural products with potential anti-MMP-2/-9 activities.

KEYWORDS

zymography, gelatinase, MMP, serralysin, anti-aging, *Serratia marcescens*, keratinocyte

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INTRODUCTION

In the past decades, a lot of effort has been made in associating matrix metalloproteinases with different physiologic processes of aging and with pathologic processes including cancer. Matrix metalloproteinases (MMPs) are a family of zinc-containing endopeptidases which are involved in the remodelling and degradation of the extracellular matrix (ECM). MMPs are divided into five subgroups according to substrate specificity, structural similarities and cellular location: Collagenases, stromelysins, gelatinases, matrilysins and membrane-type MMPs (Nagase and Woessner 1999). The sequences of more than 60 MMPs have been determined, among them the currently known 26 human MMPs (Verma and Hansch 2007). The collagenase MMP-1 cleaves type I collagen into specific fragments, which are further hydrolysed by other MMPs e.g., the gelatinases MMP-2 and MMP-9 (Shapiro 1998). The expression and activity of MMPs are tightly controlled. The proteolytic activity of MMPs is stimulated by cleavage of their propeptide domain and counteracted by natural inhibitors, the tissue inhibitors of MMPs (TIMPs) (Visse and Nagase 2003). On the other hand, regulation of MMP biosynthesis occurs primarily at the transcriptional level. In skin cells, enhanced expression of MMPs has been observed upon treatment with growth factors as well as upon exposure to UV irradiation or tumor promoters like 12-O-tetradecanoylphorbol-13-acetate (TPA) (Chua et al. 1985; Ramos et al. 2004; Steinbrenner et al. 2005). Photo-aging of the skin is related to degradation of collagen due to UV-induced expression and secretion of MMPs in dermal fibroblasts and epidermal keratinocytes (Scharffetter-Kochanek et al. 1997). The ability of MMPs to degrade macromolecular components of the ECM also accounts for their essential role in tumor invasion and cancer progression (Scharffetter-Kochanek et al. 1997; Klein et al. 2004). In addition to their influence on ECM homeostasis, more functions of MMPs have been revealed in cell growth and motility, regulation of apoptosis and modulation of chemokine bioactivity (Cauwe et al. 2007). Thus, MMPs are implicated in physiological processes including angiogenesis, wound healing, skeletal growth and remodelling, as well as degenerative and inflammatory diseases such as arthritis and multiple sclerosis (Cauwe et al. 2007).

Because of the involvement of MMPs in the initiation and progression of many human diseases, further development of potent and specific MMP inhibitors is highly warranted. Besides designing synthetic MMP inhibitors, natural products are screened for potential anti-MMP ability. Tetracyclines, catechin derivatives, pycnidione and pseudopeptides such as actinonin or matlystatin B have been identified as MMP inhibitors (Whittaker et al. 1999). Natural products containing carotenoids and polyphenols with the ability to inhibit MMPs have been reported to be effective in the prevention of pulmonary and heart diseases as well as in minimizing the risk of cancer (Sartor 2002; Cawston et al. 2001). Furthermore, many commercially available anti-aging products are designed to counteract loss of skin matrix by inhibiting the enzymatic activity of MMPs. Bioassays are routinely used for the determination and quantitation of MMPs *in vivo* and *in vitro*. In particular, the proteolytic activity of collagenases and gelatinases can be determined through a simple and sensitive assay of zymography (Catterall and Cawston 2003; Steinbrenner et al. 2003).

Presented in this study is an established gelatin zymography-based assay for pre-screening of agents with assumed anti-MMP activity, including natural products and anti-aging creams. This study made use of gelatinase-secreting *Serratia marcescens* bacteria (Maeda and Morihira 1995), which is easy and cost-effective to cultivate, thus providing a means of routine screening. The results obtained with bacterial gelatinases were subsequently validated on MMP-2 and MMP-9, gelatinases secreted by human keratinocytes.

MATERIALS AND METHODS

Reagents

For zymography, the following chemicals were used: Acrylamide/Bis solution (Bio-Rad; Hercules, CA), TEMED, gelatin (Sigma, St. Louis, MO), Triton X-100 and Brij-35. For cultivation of *Serratia marcescens* bacteria, Difco Nutrient Broth medium was used. If not stated otherwise, all reagents were obtained from Bio Basic, Markham, Canada.

Preparation of bacterial culture supernatants

Frozen stocks of *Serratia marcescens*, provided by the UST Collection of Microbial Strains (UST-CMS), were inoculated in nutrient broth and incubated at 37°C for 7-9 h. Thereafter, the bacteria were inoculated into new nutrient broth and incubated at 37°C for 18 h. Incubated bacteria were harvested and centrifuged at 12,000 rpm for 5 min. Culture supernatants containing the bacterial gelatinases were collected and stored at -80°C.

Preparation of culture supernatants from human keratinocytes

Cells were maintained at 37°C in a humidified 5% CO_2 atmosphere. Normal human epidermal keratinocytes (NHEK) were prepared from foreskin biopsies as described (Glade et al. 1996), and grown in keratinocyte-SFM medium (Invitrogen; Karlsruhe, Germany) containing supplements (human epidermal growth factor, bovine pituary extract; Invitrogen) and gentamycin (5µg/ml; Sigma). HaCaT cells, immortalised human keratinocytes (Boukamp et al. 1988), were kindly provided by Dr. N. Fusenig (German Cancer Research Center, DKFZ Heidelberg), and grown in DMEM (Sigma), supplemented with 10% fetal calf serum (PAA; Pasching, Austria), 100 U/ml penicillin, 100 µg/ml streptomycin (PAA) and 2 mM glutamax (Invitrogen). Cells were cultivated in serum-free medium for 24 h, and culture supernatants containing MMP-2 and MMP-9 gelatinases were collected and stored at -80°C.

Treatment of bacterial culture with EDTA

To verify *in vitro* that the secreted gelatinase of *Serratia*

marcescens possesses characteristics of MMPs, bacterial supernatant was treated with a chelating agent, EDTA (ethylenediaminietetraacetic acid), that is known to inhibit gelatinolytic activity (Todor et al. 1998). Aliquots of bacterial culture supernatants were incubated with different concentrations of EDTA on ice for 1 h and were then subjected to gelatin zymography.

The EDTA-treated samples were mixed and incubated on ice for 1 h to prevent denaturation of proteins while allowing inhibition to occur. After incubation, volumes equivalent to 1µg of protein were obtained from each sample and were added to 10µL loading buffer. Final volumes were all loaded in the gel. The amount of protein was determined by the Bradford method (Bradford 1976).

Pre-screening of agents for anti-gelatinolytic capacity

Vitamin E-, aloe vera- and tretinoin-containing anti-aging creams as well as natural products (extracts of banaba leaves, green papaya, tomato and guava) were screened for their ability to inhibit the activity of gelatinases in culture supernatants of *Serratia marcescens* bacteria. These commercially-available cosmetic products were chosen because of their claimed anti-aging effects and water solubilities. Anti-aging creams were solubilized in water. Maximum solubility in water of each was determined and served as stock solutions. Aqueous extracts of natural products were prepared by osteorizing each of the samples in cold water (1:1) and were then filtered. The filtrates were lyophilized and stored at 4°C until use. A stock solution of 1 mg/mL was prepared for each lyophilized sample. The chelating agent EDTA served as positive control. Equal aliquots of bacterial culture supernatants were incubated on ice for 1 h with various concentrations in serial dilution of anti-aging creams or plant extracts, and subjected to gelatin zymography thereafter. To validate the assay, this procedure was repeated using culture supernatants of human keratinocytes (NHEK and HaCaT).

Gelatin zymography

Activity of gelatinases in culture supernatants of *Serratia marcescens* bacteria or human keratinocytes was determined by gelatin zymography as described (Steinbrenner et al. 2003). Briefly, zymography was done by running aliquots of the supernatants (1 ug protein) under denaturing, but non-reducing conditions in 10% SDS-polyacrylamide gels containing 0.1% gelatin. After electrophoresis, gels were incubated for 1 h in 2.5% Triton X-100, followed by overnight incubation at 37°C in reaction buffer (50mM Tris-HCl pH 7.3, 200 mM NaCl, 5 mM $CaCl_2$, 0.02% Brij 35). Gels were stained with Coomassie brilliant blue solution for 10 min and destained with 10% acetic acid for 1 h to visualize the bands of proteolytic activity. Molecular sizes of gelatinolytic bands were calculated by comparison with a prestained protein marker (Fermentas; St. Leon-Rot, Germany).

RESULTS AND DISCUSSION

Zymography, an activity-based electrophoretic assay

The most simple, sensitive, and widely used technique for the detection of MMPs is substrate zymography, which identifies expression of MMP with regards to their ability to degrade their non-modified natural substrates (Snoek-van Beurden and Von den Hoff 2005). The standard method for zymography is based on the use of SDS-polyacrylamide gels co-polymerized with a protein substrate that may be gelatin, casein, or fibrin (Catteral and Cawston 2003). It has been used for many years mainly with gelatin. Proteins are separated by electrophoresis under denaturing (caused by SDS- sodium dodecyl sulfate), nonreducing conditions. During electrophoresis, MMPs are denatured by SDS and become inactive. After electrophoresis, the gel is incubated with Triton X-100 to remove the SDS, and the MMPs are reactivated. Overnight incubation of gels with reaction buffer at 37°C allows the reactivated enzyme to degrade the "in gel" copolymerized substrate. Subsequently, staining of gels with Coomassie Brilliant Blue solution reveals sites of proteolysis of the substrate as clear bands against a dark blue background. All types of zymography originate from gelatin zymography. The techniques involved are the same, varying only in the substrate to be used depending on the type of MMP to be detected (Snoek-van Beurden and Von den Hoff, 2005). The intensity of the proteolytic bands is linearly related to the amount of protease loaded in the gel within a certain range (Leber and Balkwill, 1997).

Detection of the *Serratia marcescens*-secreted gelatinase serralysin

The Gram-negative bacterium *S. marcescens* has been described to preferentially hydrolyze gelatin (Vermelho et al. 1996). *S. marcescens* is capable of secreting a number of enzymes including a nuclease, a chitinase, lipases and metalloproteases (Hines et al. 1988). Serralysin, the major zinc-dependent metalloprotease secreted by *S. marcescens*, possesses the conserved zinc-binding sequence motif *HEIGHALGLSHP*, which is followed by a conserved methionine residue (Baumann et al. 1998). Serralysin occurs in two isoforms with molecular weights of 50 kDa and 53 kDa (Baumann et al. 1998). By gelatin zymography, we determined the presence of these gelatinases in culture supernatants of *S. marcescens*. Consistent with former studies (Vermelho et al.1996; Baumann et al. 1998), two closely migrating gelatinolytic bands, with molecular weights around 50 kDa, from culture supernatants of *S. marcescens*, were observed in the zymogram. Next, we tested whether the activity of *S. marcescens*–secreted gelatinases can be suppressed by EDTA, a known inhibitor of human MMPs (Todor et al. 1998). This served as the positive control for the assay. Treatment of *S. marcescens* culture supernatants with EDTA caused a dose-dependent decrease of gelatinolytic activity (Figure 1). Human MMPs as well as *S. marcescens*–secreted serralysin depend on zinc ion (Zn^{2+}) for their enzymatic activity; thus, the inhibitory effect of EDTA is likely to be accounted for by its metal-chelating properties.

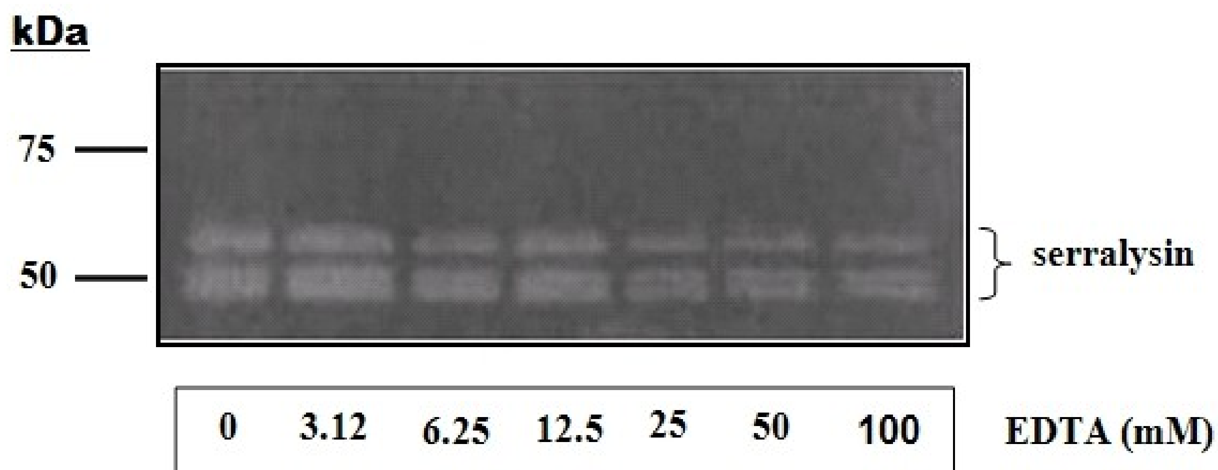

Figure 1. Effect of chelating agent EDTA on the gelatinolytic activity of *Serratia marcescens*-secreted serralysin. Bacterial supernatants were incubated for 1 h with different concentrations of EDTA and subjected to gelatin zymography thereafter. Concentrations of EDTA are indicated. The zymogram represents one out of three independent experiments.

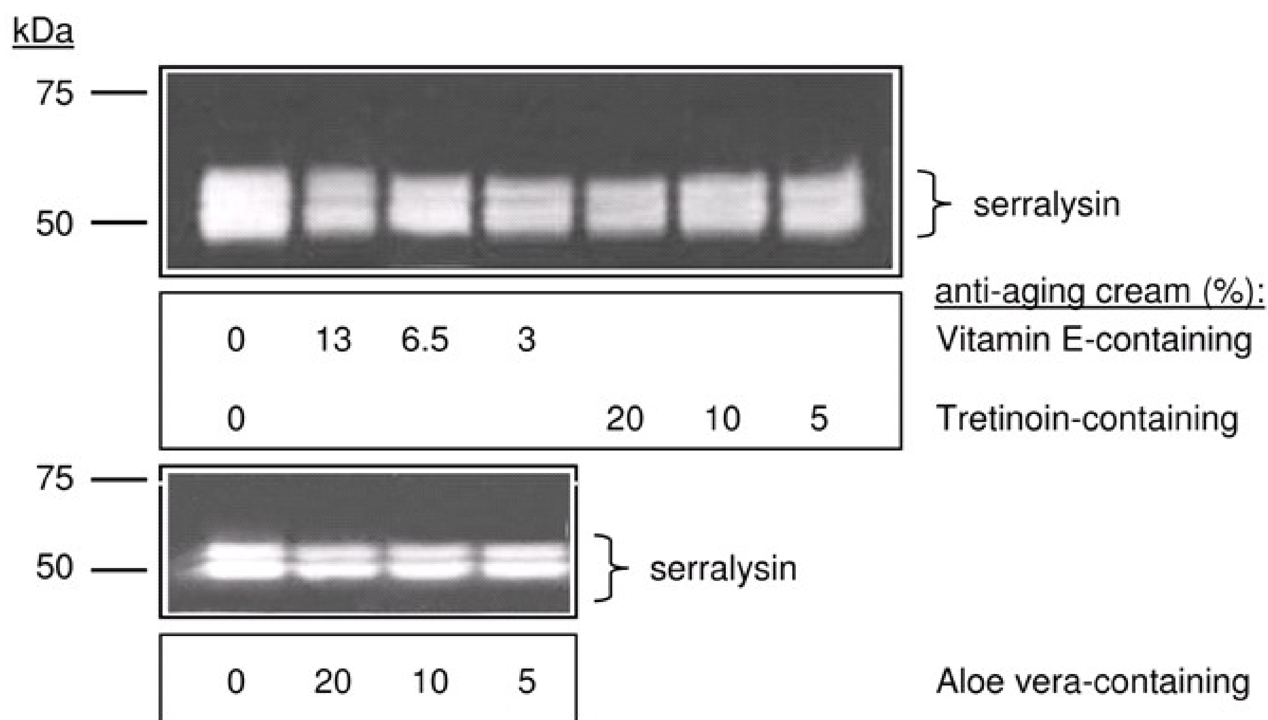

Figure 2. Effect of anti-aging creams on the gelatinolytic activity of *Serratia marcescens*-secreted serralysin. Bacterial supernatants were incubated for 1 h with diluted anti-aging creams containing vitamin E, tretinoin or aloe vera, and subjected to gelatin zymography thereafter. Concentrations of the anti-aging creams are indicated. The zymograms represent one out of three independent experiments.

Pre-screening of agents for assumed anti-gelatinolytic capacity

The established assay was used to pre-screen the anti-gelatinolytic capacity of selected commercially available anti-aging creams and aqueous extracts of natural products. Serial dilutions of water-based anti-aging creams containing vitamin E, tretinoin or aloe vera were incubated with culture supernatants of *Serratia marcescens* for 1 h on ice. Figure 2 shows the effect of the anti-aging products on the gelatinolytic activity of *S. marcescens*-secreted serralysin. We observed a marked decrease in gelatinolytic band intensities of serralysin caused by vitamin E- and tretinoin-containing anti-aging creams, whereas the aloe vera-containing anti-aging cream elicited only a slight inhibitory effect. Vitamin E is known to inhibit enzymes promoting the breakdown of collagen, and to protect cell membranes from lipid peroxidation (Lupo 2001). Tretinoin (all-trans retinoic acid) increases the production of collagen in the dermis, and has been demonstrated to inhibit the synthesis and secretion of collagenases and gelatinases in fibroblasts (Bauer et al.1983). Topical application of aloe vera leaf gels has been reported to improve wound healing and skin hydration (Hamman 2008). The tested anti-aging products include additional ingredients which might contribute to the observed anti-gelatinolytic capacity: pro-vitamin B5, glycerine, dimethicone and/or hydroquinone. Moisturizers containing dimethicone and glycerine have been applied to increase epithelial thickness and barrier function in photoaged skin (Short et al. 2007). Hydroquinone has been demonstrated to inhibit MMP-2 activity *in vitro* (Alcazar et al. 2007).

In another set of experiments, serial dilutions of aqueous extracts of green papaya, tomato, guava and banaba leaves were

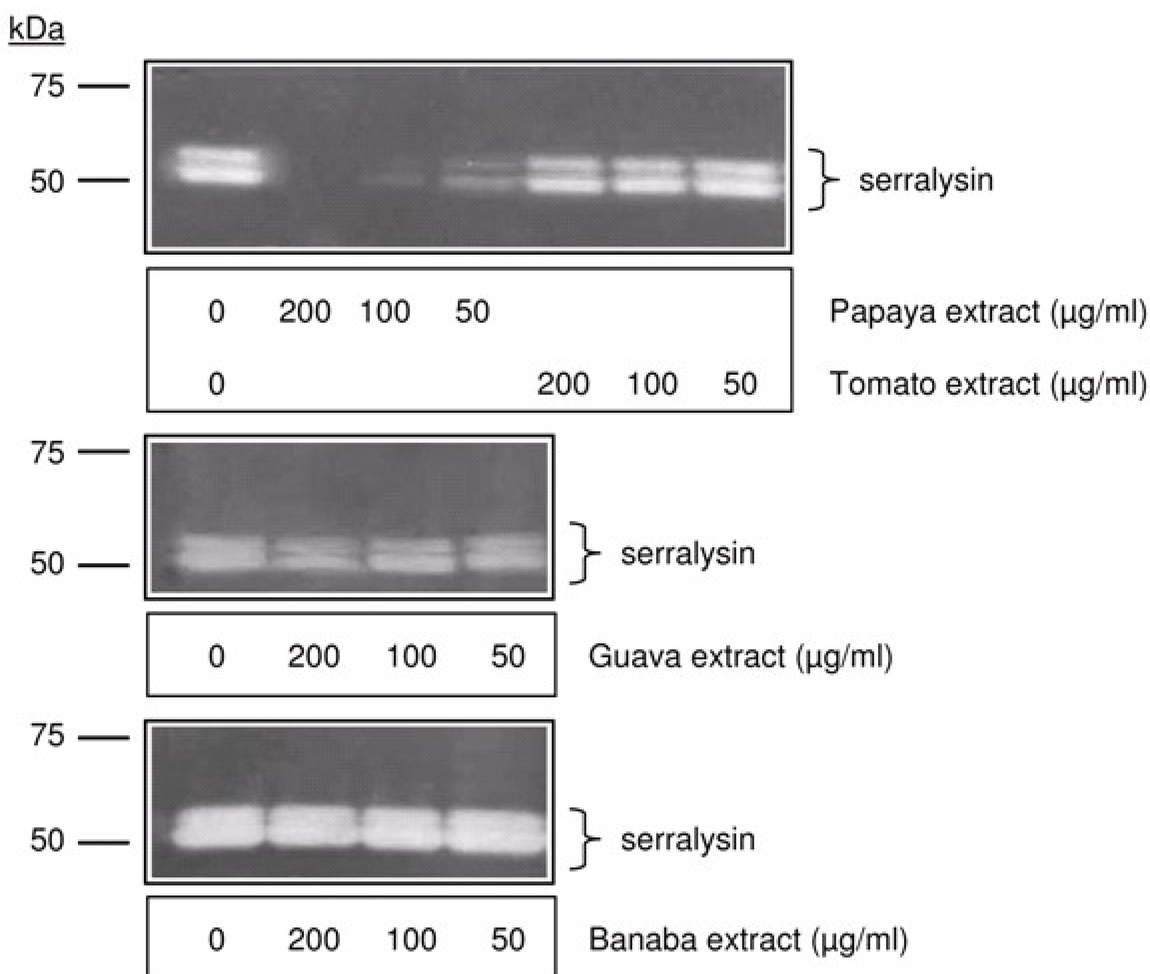

Figure 3. Effect of aqueous extracts of papaya, tomato, guava and leaves of banaba on the gelatinolytic activity of *Serratia marcescens*-secreted serralysin. Bacterial supernatants were incubated for 1 h with plant extracts at the indicated concentrations, and subjected to gelatin zymography thereafter. The zymograms represent one out of three independent experiments.

tested. As shown in Figure 3, we observed a strong dose-dependent inhibition of the gelatinolytic activity of *S. marcescens*-secreted serralysin by papaya extracts. Furthermore, tomato and guava extracts were capable of inhibiting the gelatinolytic activity of serralysin, though to a lesser extent. In contrast, extracts of banaba leaves did not show any anti-gelatinolytic capacity in this assay. Papaya (*Carica papaya)* is a tropical plant, whose fruits contain large amounts of the proteolytic enzyme papain along with phytochemicals such as β-carotene, thiamine, riboflavin, niacin, and vitamins C and E. The strong inhibitory effect of papaya extract in the zymography assay can most likely be explained by the ability of papain to efficiently hydrolyse proteins containing cysteine residues. Both tomatoes and guava fruits are rich in bioactive compounds including anti-oxidants and vitamins, which may account for their anti-gelatinolytic capacity in this assay. Tomato (*Lycopersicon esculentum*) contains the carotenoid lycopene, an oral sun protectant, which has been demonstrated to ameliorate erythema caused by ultraviolet (UV) irradiation (Gärtner et al. 1997; Heinrich et al. 2003). In guava (*Psidium guajava*), different carotenoids, retinol, vitamin B1 and B2, niacin and vitamin C have been found. Leaves of banaba (*Lagerstroemia speciosa*) are traditionally used in Philippine herbal medicine for treatment of diabetes and obesity; corosolic acid and gallotannins have been identified as the responsible bio-active compounds (Klein et al. 2007). However, we did not observe an anti-gelatinolytic effect of extracts of banaba leaves in the zymography based on serralysin.

As for the negative control, culture supernatants of *Serratia marcescens* were incubated with different concentrations of *Kielmeyera coriacea* extracts. Figure 4 shows that aqueous extract of *K. coriacea* did not exhibit anti-gelatinolytic acitivity against *Serratia marcescens* serralysin. *Kielmeyera coriacea,* commonly known as “Pau Santo” or “Saco de Boi”, is found in central Brazil and up to date, there is still no clear evidence or study that extracts of *K. coriacea* show anti-MMP activity. A study made by Alves et al. (2000) claimed that the aqueous extract of *K. coriacea* is used in popular folk medicine for treatment of several tropical diseases, including schistosomiasis, leishmaniasis, malaria, and fungal and bacterial infections. Previous studies have shown that phytochemicals, xanthones, terpenes and biphenyl are present in extracts of *K. coriacea* (Cortez et al. 1998). In a study made by Obici and collaborators (2008), dichloromethane extracts of *K. coriacea* stem appeared to have no toxicity in rats after oral acute administration.

Comparison of the serralysin-based pre-screening with a zymography assay for human gelatinases

Finally, we evaluated whether the agents with demonstrated anti-gelatinolytic capacity for serralysin are capable of inhibiting human gelatinases as well. Fibroblasts, keratinocytes and endothelial cells in the skin produce various amounts of MMP-2 (72 kDa gelatinase) and MMP-9 (92 kDa gelatinase) (Oikarinen et al. 1993). Herein, we used normal human epidermal keratinocytes (NHEK) and a human keratinocyte cell line (HaCaT). Consistent with previous studies (Steinbrenner et al. 2003; Kobayashi et al. 2000), high gelatinolytic activity of MMP-9 and weak activity of MMP-2 were observed in culture supernatants of NHEK, whereas HaCaT cells only secreted MMP-2 (Figures 5 and 6). The capacity of the tested commercial anti-aging products to act as inhibitors of human MMPs (Figures 5A and 5B) was in accordance with the results of pre-screening in the serralysin-based assay: The aloe vera-containing anti-aging cream, pre-screened as a very weak inhibitor for serralysin (Figure 2), was not capable of affecting the activity of MMP-2

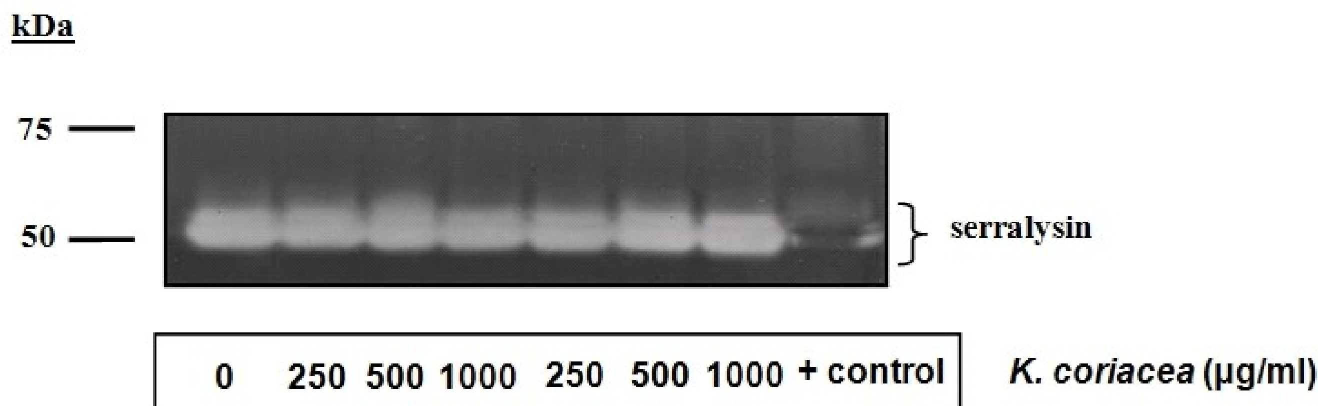

Figure 4. Effect of aqueous extracts of *Kielmeyera coriacea* on the gelatinolytic activity of *Serratia marcescens*-secreted serralysin. Bacterial supernatants were incubated for 1 h with plant extracts at the indicated concentrations, and subjected to gelatin zymography thereafter. Aqueous extract of papaya was used as positive control. The zymogram represents one out of three independent experiments.

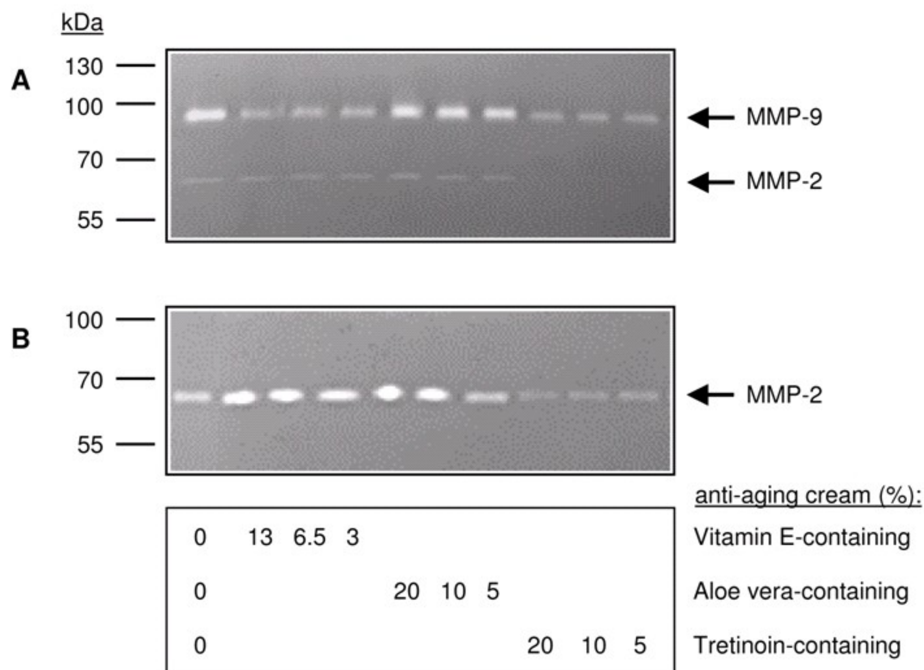

Figure 5. Effect of anti-aging creams on the activity of the gelatinases MMP-2 and MMP-9 secreted by keratinocytes. Culture supernatants of serum-starved normal human epidermal keratinocytes (NHEK) **(A)** and the human keratinocyte cell line HaCaT **(B)** were incubated for 1 h with diluted anti-aging creams containing vitamin E, tretinoin or aloe vera, and subjected to gelatin zymography thereafter. Concentrations of the anti-aging creams are indicated. The zymograms represent one out of three independent experiments.

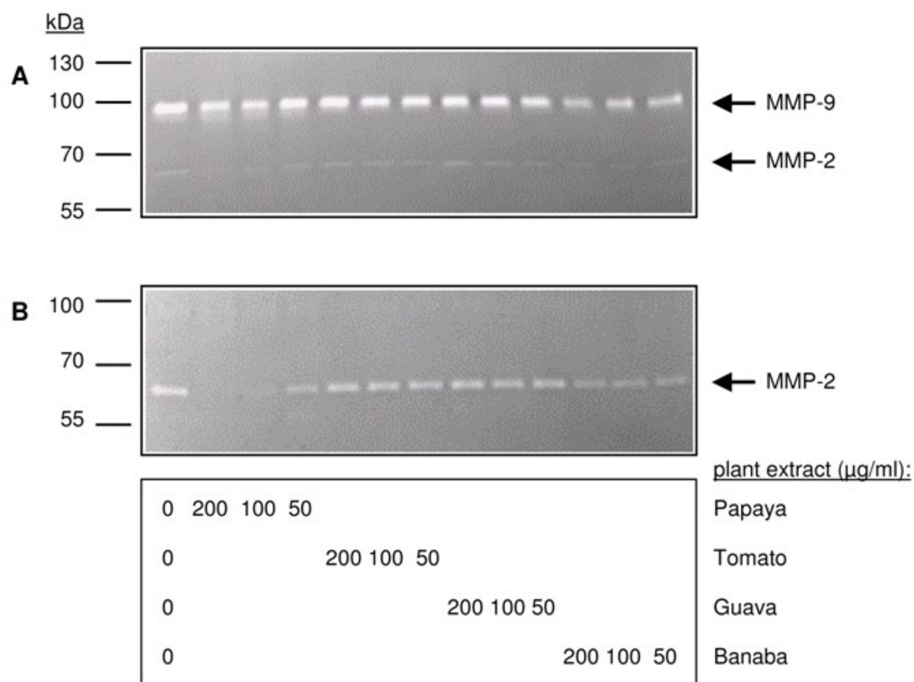

Figure 6. Effect of aqueous extracts of papaya, tomato, guava and leaves of banaba on the activity of the gelatinases MMP-2 and MMP-9 secreted by keratinocytes. Culture supernatants of serum-starved NHEK **(A)** and HaCaT **(B)** were incubated for 1 h with plant extracts at the indicated concentrations, and subjected to gelatin zymography thereafter. The zymograms represent one out of three independent experiments.

or MMP-9. Strikingly, vitamin E- and tretinoin-containing creams, pre-screened as effective gelatinase inhibitors in the serralysin-based assay, strongly decreased the gelatinolytic activity of MMP-9. The tretinoin-containing anti-aging cream additionally inhibited the gelatinolytic activity of MMP-2. In contrast to other anti-oxidants such as N-acetylcysteine (NAC), which have been shown to inhibit the gelatinolytic activities of both MMP-2 and MMP-9 (Steinbrenner et al. 2005), the vitamin E-containing cream did not affect MMP-2 despite its remarkable inhibition of MMP-9 (Figure 2).

Among the tested plant extracts, papaya exhibited the strongest anti-gelatinolytic capacity (Figure 6). As observed for serralysin before (Figure 3), papaya extract dose-dependently decreased the activities of human gelatinases MMP-2 and MMP-9, most probably due to the included enzyme papain. In contrast, the inhibition of serralysin by tomato and guava extracts could not be confirmed in the zymography for MMP-2 and MMP-9. Furthermore, the extract of banaba leaves, which did not show any anti-gelatinolytic capacity in the serralysin-based assay, was an efficient inhibitor of both human MMP-2 and MMP-9 (Figures 6A and 6B). We used the same working concentrations for all tested plant extracts in the zymography assays with serralysin as well as with MMP-2 and MMP-9; thus, further experiments are required to ascertain whether the observed discrepancies are due to dose-dependent effects or may derive from different modes of inhibition.

Taken together, the established zymography assay based on the bacterial gelatinase serralysin has been demonstrated to be a reliable tool, which may be applied for the fast and cost-effective pre-screening of assumed inhibitors of matrix metalloproteases. The ability of several tested substances to inhibit serralysin and the human gelatinase MMP-9 at similar doses appears to be particularly interesting, because MMP-9 contributes to keratinocyte hyperproliferation, represents one of the key enzymes associated with tumor progression and is a marker of malignant transformation in various human cancers (Coussens et al. 2000; Dechow et al. 2004; Jordan et al. 2004).

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CONTRIBUTIONS OF INDIVIDUAL AUTHORS

This research work was part of the thesis for the M.Sc. degree of J.N.B., under the advisorship of M.C.R. H.S. tested the inihibitory activity of the samples against MMP-2/MMP-9 secreted by NHEK and HaCaT cell line.

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