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MMP Activity in the Hybrid Layer Detected with *in situ* Zymography

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ABSTRACT

Dentinal proteases are believed to play an important role in the degradation of hybrid layers (HL). This study investigated the HL gelatinolytic activity by *in situ* zymography and functional enzyme activity assay. The hypotheses were that HLs created by an etch-and-rinse adhesive exhibit active gelatinolytic activity, and MMP-2 and -9 activities in dentin increase during adhesive procedures. Etched-dentin specimens were bonded with Adper Scotchbond 1XT and restored with composite. Adhesive/dentin interface slices were placed on microscope slides, covered with fluorescein-conjugated gelatin, and observed with a multi-photon confocal microscope after 24 hrs. Human dentin powder aliquots were prepared and assigned to the following treatments: A, untreated; B, etched with 10% phosphoric acid; or C, etched with 10% phosphoric acid and mixed with Scotchbond 1XT. The MMP-2 and -9 activities of extracts of dentin powder were measured with functional enzyme assays. Intense and continuous enzyme activity was detected at the bottom of the HL, while that activity was more irregular in the upper HL. Both acid-etching and subsequent adhesive application significantly increased MMP-2 and -9 activities ($p < 0.05$). The results demonstrate, for the first time, intrinsic MMP activity in the HL, and intense activation of matrix-bound MMP activity with both etching and adhesive application.

KEY WORDS: human dentin, dentin bonding agent, degradation, MMP-2, MMP-9, biochemical assays.

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INTRODUCTION

There is a general consensus that resin-dentin bonds created with contemporary hydrophilic dentin bonding systems deteriorate over time (Breschi *et al*., 2008; Liu *et al*., 2011). For etch-and-rinse adhesives, there is a decreasing gradient of resin monomer diffusion within the hybrid layers (Wang and Spencer, 2002; Breschi *et al*., 2004). This results in incomplete resin infiltration at the bottom of the hybrid layer (HL) that contains denuded collagen fibrils (Armstrong *et al*., 2001; Breschi *et al*., 2004). Enzymatic degradation of these partially non-impregnated collagen fibrils has been suggested to occur due to the activation of their collagen-bound matrix metalloproteinases (MMPs) (Pashley *et al*., 2004; Mazzoni *et al*., 2006; Nishitani *et al*., 2006; De Munck *et al*., 2009; Osorio *et al*., 2011).

Human dentin contains at least MMP-2, -9 (gelatinases) (Martin-De Las Heras *et al*., 2000; Mazzoni *et al*., 2007, 2009; Toledano *et al*., 2010), -8 (collagenase) (Sulkala *et al*., 2007), -3 (stromelysin) (Boukpessi *et al*., 2008; Mazzoni *et al*., 2011), and -20 (enamelysin) (Sulkala *et al*., 2002). Since endogenous dentin MMPs are bound to collagen fibrils in mineralized dentin (Mazzoni *et al*., 2009), acid-etching causes MMP exposure, and the subsequent application of acidic monomers in etch-and-rinse or self-etch adhesives increases the enzyme activity (Mazzoni *et al*., 2006; Nishitani *et al*., 2006; Tay *et al*., 2006; De Munck *et al*., 2009; Osorio *et al*., 2011).

Despite all previous studies, direct evidence of the activity of endogenous dentin MMPs within resin-bonded HLs is lacking. The aim of this study was to assess the gelatinolytic activity of an adhesive interface using an *in situ* zymographic technique and functional enzyme activity assay, allowing for the specific quantitation of gelatinolytic MMP-2 and -9. The tested hypotheses, based on a previous experiment with powdered dentin and etch-and-rinse adhesives (Mazzoni *et al*., 2006), were (1) that HLs created with an etch-and-rinse adhesive exhibit endogenous gelatinolytic activity, and (2) that adhesive procedures increase MMP-2 and -9 activities in HLs.

MATERIALS & METHODS

Reagents were purchased from Sigma Chemical (St. Louis, MO, USA) unless otherwise specified.

In situ Zymography of the Hybrid Layer

Fifteen freshly extracted non-carious human third molars were used in this study, which was approved by the Ethical Committee of the University of Trieste, Italy. After removal of enamel and cementum, 1-mm-thick disks of middle/deep coronal dentin were obtained from each tooth by means of a slow-speed saw (Micromet, Remet, Casalecchio di Reno, Italy). A standardized smear layer was created with 600-grit wet silicon-carbide paper, and dentin was etched for 15 sec with 35% phosphoric-acid gel (3M ESPE, St. Paul, MN, USA) and rinsed with continuous water irrigation for 30 sec. Adper Scotchbond 1XT adhesive (3M ESPE) was applied on acid-etched dentin in accordance with the manufacturer's instructions. A 1-mm-thick flowable composite (Filtek Flow; 3M ESPE) was applied to bonded disks and light-cured for 20 sec with a quartz-tungsten-halogen light-curing unit (Curing Light 2500, 3M ESPE). Bonded specimens were then cut vertically into 1-mm-thick slabs to expose the adhesive/dentin interfaces by means of a slow-speed saw (Micromet).

Each bonded dentin/composite site was glued to a microscope slide with cyanoacrylate cement and ground down to obtain *ca*. 500-μm-thick specimens. *In situ* zymography was performed with quenched fluorescein-conjugated gelatin as the MMP substrate (E-12055, Molecular Probes, Eugene, OR, USA). A 1.0 mg/mL stock solution of fluorescein-labeled gelatin was prepared by the addition of 1.0 mL water to the vials containing the lyophilized substrate that was stored at −20°C until used. The gelatin stock solution was diluted 1:8 with the dilution buffer (NaCl 150 mM, $CaCl_2$ 5 mM, Tris-HCl 50 mM, pH 8.0), and an anti-fading agent was added (Mounting Medium with Dapi H-1200, Vectashield, Vector Laboratories LTD, Cambridgeshire, UK). A 50-μL quantity of the fluorescent gelatin mixture was placed on top of each slab and covered with a coverslip. Slides were light-protected and incubated in humidified chambers at 37°C. For identification of the optimum incubation period, fluorescent images were made from 1 hr to 7 days. Detailed description of the 3D analysis of *in situ* zymography with confocal microscopy is provided in the Appendix. Briefly, hydrolysis of quenched fluorescein-conjugated gelatin substrate, indicative of endogenous gelatinolytic enzyme activity, was assessed by examination under a multi-photon confocal microscope, *ex*:488nm and *em:*lp530nm (Zeiss, LSM 780, Carl Zeiss, Oberkochen, Germany). Optical sections of 85 μm thick were acquired from different focal planes, and the stacked images were analyzed, quantified, and processed with ZEN 2010 software (Carl Zeiss).

Negative control sections were incubated as described above except that (1) 250 mM ethylenediaminetetraacetic acid (EDTA) was dissolved in the mixture of quenched fluorescein-conjugated gelatin (Sakuraba *et al*., 2006) or (2) standard non-fluorescent instead of fluorescent-conjugated gelatin was used.

Assay to Determine MMP-2 and -9 Activities

Twelve additional human teeth were used for the determination of MMP-2 and -9 activities. Dentin powder was obtained as described previously (Mazzoni *et al*., 2007). Aliquots of 250 mg of mineralized dentin powder were prepared and assigned to one of the treatment groups (n = 3 *per* group): A, untreated mineralized powder; B, powder acid-etched with 10% phosphoric acid for 1 min and then rinsed in water to simulate partial dentin demineralization; or C, acid-etched as in group B, and then mixed for 1 min with Adper Scotchbond 1XT; the unpolymerized adhesive was then removed by acetone extraction (10 mL for 5 min) and centrifugation (20,000 *g*). Re-suspension in acetone and re-centrifugation were performed 3 times for complete removal of the resin monomers.

The activities of MMP-2 and -9 were determined with the Biotrak™ activity assay system (GE Healthcare, Buckinghamshire, UK). Protein extraction was performed in 50 mM Tris-HCl buffer, pH 7.4, clarified by centrifugation (275 *g* for 10 min), and supernatants were assayed for MMP-2 and -9 activities separately. Standard curves were prepared, and samples were incubated in the supplier-provided assay buffer for 12 hrs at 4°C. After extensive rinses, the detection reagent was added and absorbancies read at 405 nm (BioRad, Segrate Milano, Italy). Assays were performed in triplicate according to the manufacturer's instructions. Since values were normally distributed (Kolmogorov-Smirnof test), data were analyzed with one-way ANOVA and Tukey's *post hoc* test ($p < 0.05$).

RESULTS

In situ Zymography of the HL

For all the assayed specimens, the *in situ* zymography revealed an intense green fluorescence in mineralized dentin and within the HL after 24 hrs of incubation, indicating that the fluorescein-conjugated gelatin was strongly hydrolyzed at these sites (Fig. 1A). The overlay image activity was most intense in the dentinal tubules and at the bottom of the HL as a 1- to 2-μm-thick, relatively well-defined, layer (Fig. 1C), representing the partially demineralized, poorly resin-infiltrated collagen matrix. The intensity of the fluorescence did not increase after 24-hour incubation.

No fluorescence was detected in negative controls, *i.e*., both (1) EDTA-treated (data not shown) and (2) specimens incubated with standard non-fluorescent gelatin (Figs. 1D-1F).

The fluorescence intensity emitted by hydrolyzed fluorescein-conjugated gelatin was quantified (Figs. 1G, 1H) and compared with the control sample by assessment of the specific emitted fluorescence (Fig. 1I), as well as by the relative fluorescent area in each panel (Fig. 1J).

An example of how a sequence of acquired images of the adhesive/dentin interface is obtained by multi-photon confocal microscopy in different focal planes is shown in Fig. 2A. All focal planes were recorded and can be accessed in the Appendix. Fig. 2B shows, in the Z-axis, the presence of infiltrated fluorescent gelatin in all acquired focal planes. The 3-D model of the

acquired stacked images confirmed the intense and continuous gelatinolytic activity at the bottom of the HL (Fig. 3A). In addition, more intermittent activity was present in the HL closer to the adhesive-composite interface. In mineralized dentin, the activity was localized mostly at the inner tubular periphery (Fig. 3B).

MMP-2 and -9 Activities

With the untreated mineralized dentin powder used as control, both MMP-2 and -9 activities increased significantly after acid etching compared with the control (untreated dentin powder; $p < 0.05$) (Table). Further significant increase was again seen after adhesive treatment compared with the control or acid-etched dentin ($p < 0.05$) (Table).

DISCUSSION

Several studies have demonstrated the presence and activity of endogenous proteolytic enzymes in human dentin, but no one has been able to evaluate the proteolytic activity in HLs directly *in situ*. The primary obstacle to assaying the proteolytic activity of HLs relies on the methods available to detect, precisely localize, and measure enzyme activities in whole tissue, because most methods require that the proteolytic enzymes be extracted from the tissues prior to analysis. Since the HL is a microscopic biological composite derived from resin infiltration of a tissue with a proteolytic activity (*i.e.*, dentin) (Tjäderhane *et al*., 1998; Pashley *et al*., 2004; Mazzoni *et al*., 2007), these commonly used methods to verify the presence and activity of enzymes are not possible. Even so, for better understanding and perhaps prevention of the proteolytic degradation of resin-dentin bonds, identification of the mechanisms involved in the exposure and activation of these enzymes in the dentin-adhesive interface is essential. This report is the first time anyone has assayed for the endogenous proteases of the HL by *in situ* zymography, showing obvious

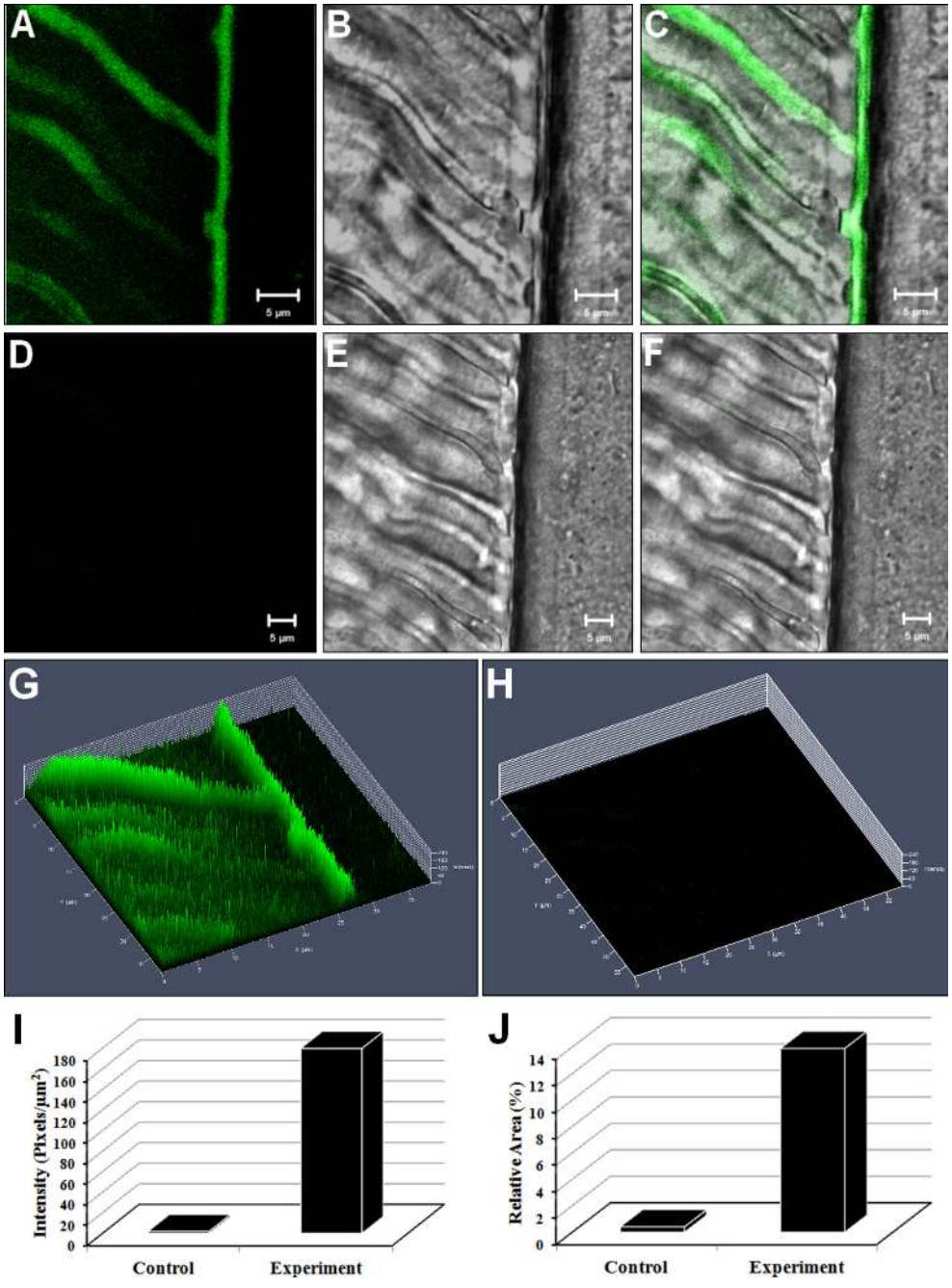

Figure 1. Resin-bonded dentin interface prepared with Adper Scotchbond 1XT and incubated for 24 hrs with quenched fluorescein-labeled gelatin (A-C) or standard non-fluorescent gelatin (negative control) (D-F); D = Dentin; HL = Hybrid layer; R = Resin Composite; bar = 5 µ. **(A)** Acquired image in green channel, showing fluorescence in dentinal tubules and within the hybrid layer (HL). **(B)** Differential interference contrast (DIC), showing the optical density of the resin-dentin interface. **(C)** Merged images A and B. **(D)** Acquired image at green channel, showing that no background fluorescence was detectable in the negative control specimen. **(E)** Differential interference contrast (DIC), showing the optical density of the interface resin-dentin in the negative control. **(F)** Merged images D and E. **(G)** Quantitative analysis of emitted fluorescence intensity in Panel A. **(H)** Quantitative analysis of emitted fluorescence intensity in control Panel D. **(I)** Specific fluorescence emission evaluation in control and experimental sample. **(J)** Relative percentage area of emitted fluorescence in control and experimental samples.

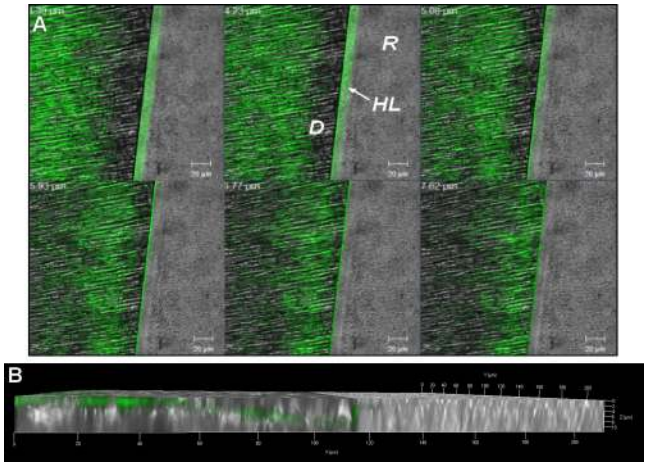

Figure 2. Sequence of acquired images in the green channel of the multi-photon confocal microscope superposed on images obtained with differential interference contrast (A, B). **(A)** An intense fluorescence was seen within the hybrid layer and within the underlying mineralized dentin at different focal depths (*i.e.*, 3.39, 4.23, 5.08, 5.93, 6.77, 7.62 μm below the surface). The shallow depths reveal relatively uniform gelatinolytic activity, while the deeper depths show activity mainly at the base of the HL. **(B)** Shows the presence of hydrolyzed fluorescent-gelatin within all focal planes at Z axis. R = resin composite, HL = Hybrid layer, D = Dentin.

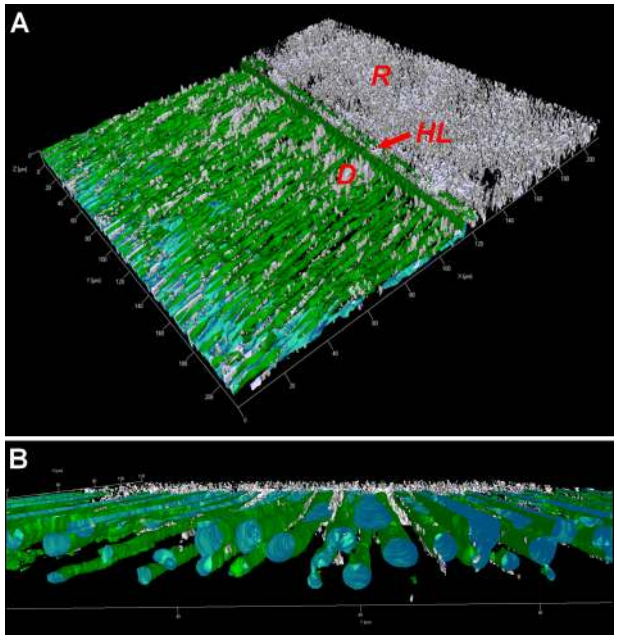

Figure 3. Three-dimensional model of the acquired image (A, B). **(A)** Shows the intense fluorescence, meaning gelatin hydrolysis, throughout the entire extension of hybrid layer. **(B)** Higher-magnification-image model shows a gelatinolytic activity inside dentinal tubules shown as cylindrical tubes, in deep dentin. The high tubule density reveals this to be very deep dentin. R = resin composite, HL = Hybrid layer, D = Dentin.

gelatinolytic activity within HLs created with a two-step etch-and-rinse adhesive. Analysis of the data indicated that host-derived gelatinases remain localized within the HL and active after bonding treatments, allowing for acceptance of the first hypothesis. The location of the activity correlates well with the demineralized uninfiltrated collagen layer simplified etch-and-rinse adhesives at the bottom of the HL (Breschi *et al*., 2004), an area also known for nanoleakage (Sano *et al*., 1995). The intermittent activity in the upper parts of the HL also correlates well with the location of partially exposed collagen detected with the highly sensitive immunogold labeling technique (Breschi *et al*., 2004) and by confocal microscopy (Kim *et al*., 2010a,b).

The absence of fluorescence in EDTA-treated samples indicates that the gelatinolytic activity is due to Ca^{++}-sensitive MMPs, and the best candidates for the enzymes are MMP-2 and -9, both detected in mineralized human dentin (Martin-De Las Heras *et al*., 2000; Mazzoni *et al*., 2007). This is also suggested by the functional specific MMP-activity assay results. The exposure of dentin to low pH (between 2.3 and 4.5) followed by neutralization (such as in caries) can significantly increase MMP-2 and -9 activation (Davis, 1991; Okada *et al*., 1995; Tjäderhane *et al*., 1998; Chaussain-Miller *et al*., 2006). The quantitative ELISA activity assay showed that endogenous dentin MMP-2 and -9 activities significantly increased after the application of a two-step etch-and-rinse adhesive compared with activities in mineralized or demineralized dentin, leading to the acceptance of the second hypothesis. The significant increase in MMP activities clearly indicates that enzymatic activation in the HL is a two-step process: Dentin demineralization and exposure of the collagen significantly increase the activity, with further significant increases after adhesive application. Thus, the acidity of Adper Scotchbond 1XT (pH 3.6) and other adhesives could be the cause of MMP activation during dentin hybridization (Nishitani *et al*., 2006; Tay *et al*., 2006; Mazzoni *et al*., 2006). In fact, adhesive-induced activation of dentinal MMPs increases in linear fashion with the decreasing pH of adhesives (Mazzoni *et al*., 2006). Together with the intense gelatinolytic activity at the bottom of the HL observed in *in situ* zymography, these findings indicate that this two-step enzyme activation process is the reason for the relatively rapid loss of dentin bond strength observed with two-step etch-and-rinse systems, both *in vivo* (Hebling *et al*., 2005; Carrilho *et al*., 2007b) and *in vitro* (García-Godoy *et al*., 2007; Carrilho *et al*., 2007a). The MMP-2 and -9 activity of demineralized dentin powder suggests that the gelatinolytic activity observed in the HL comes primarily from intertubular dentin.

Immunohistochemistry is routinely used to localize proteases in tissues (Frederiks and Mook, 2004). While immunohistochemistry allows for localization of specific MMPs, it does not provide any information on enzymatic activity. Since most of the enzymes are synthesized as being in inactive or proenzyme form (also called zymogen), which cannot usually be immunohistochemically differentiated from active proteases (Frederiks and Mook, 2004), *in situ* zymography was used, for the first time, in the present study to overcome this limitation. Our results showed that *in situ* zymography with quenched fluorescent-labeled gelatin is suitable and precise enough to investigate the involvement of proteases in HL degradation.

Table. Results of the Biotrak™ Activity Assay of Active-MMP-2 and -9 (expressed as ng/mg) in the Different Tested Groups

	Activity (ng/mg)	
Description	MMP-2	MMP-9
A: untreated mineralized dentin	3.35 ± 0.02[a]	2.41 ± 0.01[A]
B: 10% H_3PO_4 demineralized dentin	4.18 ± 0.01[b]	2.73 ± 0.02[B]
C: 10% H_3PO_4 demineralized dentin + Adper Scotchbond 1XT	4.82 ± 0.03[c]	3.58 ± 0.01[C]

[a,A]Differences between group A and groups B and C ($p < 0.05$).
[b,B]Differences between group B and groups A and C ($p < 0.05$).
[c,C]Differences between group C and groups A and B ($p < 0.05$).

The intense gelatinolytic activity in the inner tubular walls is localized either in the protein layer lining the wall or in peritubular dentin, where these proteins have been suggested to participate in the regulation of peritubular dentin formation (Hannas *et al*., 2007). The latter is more unlikely, since MMPs in mineralized dentin fraction are believed to be protected by the mineral phase, and thus they should not have access to the substrate in the protocol used in this study. The activity may also represent that of dentinal fluid MMPs precipitating into the inner wall during the processing of the samples (Tjäderhane *et al*., in press).

In conclusion, this study demonstrated that the collagen matrix within incomplete or absent resin infiltration (Breschi *et al*., 2004) possesses marked protease activity. This activity may lead to rapid loss of collagen fibrils at the bottom of the HL, as suggested in *in vitro* studies (Carrilho *et al*., 2007a; Toledano *et al*., 2007). This would significantly increase dentinal fluid influx into the HL, increasing hydrolytic degradation of both adhesive (Carrilho *et al*., 2005) and collagen (García-Godoy *et al*., 2007) in the upper parts of the HL. Eventually, the end result is the complete destruction of the HL and the loss of dentinal bond strength, as observed *in vivo* (Brackett *et al*., 2007; Carrilho *et al*., 2007b). Since both quantitative and qualitative differences in the presence of the exposed collagen in the HL exist between the adhesive systems (Breschi *et al*., 2004), *in situ* zymography combined with the functional protease activity assays would be a powerful tool to examine the differences in enzymatic activation and activities in the HL between different adhesives and adhesive systems, including one- and two-step self-etch adhesives, three-step etch-and-rinse adhesives, and glass-ionomer cements. *In situ* zymography may also permit evaluation of the relative effects of different etching times or acid concentrations on the enzymatic activity in the HL. In addition, the *in situ* technique would allow for evaluation of the effectiveness of different approaches to inhibit the enzymatic activity in the HL, which would help to develop new techniques to improve HL durability.

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