Published in final edited form as:

Acta Biomater. 2018 April 15; 71: 330–338. doi:10.1016/j.actbio.2018.02.020.

Esterase from a Cariogenic Bacterium Hydrolyzes Dental Resins

Bo Huang^{1,2}, Walter Siqueira³, Dennis G. Cvitkovitch^{1,2}, and Yoav Finer^{1,2}

¹Faculty of Dentistry, University of Toronto, Ontario, Canada

²Institute of Biomaterials and Biomedical Engineering, University of Toronto

³School of Dentistry and Department of Biochemistry, Schulich School of Medicine & Dentistry, Western University

Abstract

Objectives—To identify and characterize specific esterases from *S. mutans* with degradative activity toward methacrylate-based resin monomers.

Methods—Out of several putative esterases, an esterase encoded in an Open Reading Frame as SMU_118c (The National Center for Biotechnology Information, NCBI), was found to have true hydrolase activities. SMU_118c was cloned, expressed, purified and further characterized for its respective hydrolytic activity towards ester-containing nitrophenyl substrates and the universal resin monomers bis-phenyl-glycidyl-dimethacrylate (bisGMA) and triethyleneglycol dimethacrylate (TEGDMA) at neutral (7.0) or cariogenic (5.5) pH. Mass spectrometry (MS) was used to verify the expression of SMU_118c protein in *S. mutans* UA159.

Results—Similar to the whole cell activity of *S. mutans*, SMU_118c showed the highest affinity toward p-nitrophenyl acetate (pNPA) and p-nitrophenyl butyrate (pNPB) vs. o-nitrophenyl butyrate (oNPB) and butyrylthiocholine iodide (BTC) ($p < 0.05$). The esterase retained 60% of its activity after 21 days and hydrolyzed bisGMA at a higher rate than TEGDMA at both neutral and cariogenic pH ($p < 0.001$), similarly to the predominant human salivary esterase degradative activity. MS confirmed that SMU_118c is an intracellular protein in *S. mutans* UA159 and expressed under pathogenic (pH 5.5) growth conditions.

Significance—The similarity in the activity profile to the whole *S. mutans* bacterial cell, the stability over time at cariogenic pH, the preference to hydrolyze bisGMA and confirmed expression profile suggest that SMU_118c could be a significant contributor to the whole bacterial degradative activity of *S. mutans* toward the degradation of resin composites, adhesives and the restoration-tooth interface, potentially accelerating restoration's failure.

Graphical Abstract

Corresponding author: Dr. Yoav Finer, Faculty of Dentistry, 124 Edward Street, Toronto, Ontario, Canada, M5G 1G6, Phone: 416-864-8148, Fax: 416-979-4760, yoav.finer@dentistry.utoronto.ca.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Streptococcus mutans; esterase; resin monomers; hydrolysis; biodegradation; dental caries

1. Introduction

Resin composites are the most popular restorative materials in dentistry [1]. However, compared to amalgam, resin composites suffer from high failure rates [2, 3]; nearly 70% of resin composite restorations placed annually are replacements of failed restorations [4, 5]. One of the primary reasons for composite restoration replacement is secondary caries, defined as the progression of decay at a compromised restoration-tooth interface [6–8]. Previous studies have shown that the biostability of restorative materials have a significant impact on the quality of the restoration-tooth interface that could impact the prevalence of secondary caries [9–11].

Resin composites are inherently prone to hydrolysis due to the presence of unprotected ester linkages in their major matrix monomers bis-phenyl glycidyl dimethacrylate (bisGMA) and triethylene glycol dimethacrylate (TEGDMA), generating degradation by-products bishydroxy-propoxy-phenyl-propane (BisHPPP), triethylene glycol (TEG), and methacrylic acid (MA) respectively [12, 13]. In turn, these by-products negatively affect host cells and the metabolism and function of oral microorganisms [14–18]. It has been reported that bisphenol-A-derived species are estrogenic [19], and that the degradation by-products of TEGDMA such as MA and TEG modulate the growth of oral bacteria [14]. These degradation processes compromise the resin-tooth interface [11, 13], allowing for the ingress of cariogenic bacteria along the interface, which leads to initiation and progress of secondary caries [10, 20].

Esterases (EC 3.1.1.X) are a diverse group of hydrolases, catalyzing the hydrolysis of ester bonds. They are widely distributed in human tissues [12] and microorganisms [21, 22]. In the oral environment, the sources of esterases include human gingival epithelium, salivary glands, inflammatory responses, and microorganisms [22, 23]. Previous studies reported that human saliva contains cholesterol esterase-like (CE) and pseudocholinesterase (PCE) activities in levels that degrade resin-based composites and the restoration-tooth interface [10–13, 24–30]. It has been known that esterases are widespread among oral bacterial

pathogens such as *Streptococcus* species [31, 32] and esterases from the non-oral pathogens *Clostridium botulinum* and *Clostridium hathewayi* were reported to degrade polyesters [33, 34]. However, to date, the identification of specific esterases from cariogenic bacteria and their association with the performance and biodegradation process of resin composites and adhesives have not been investigated.

We previously reported on hydrolytic activity from the cariogenic bacterium *S. mutans* [35]. This activity profile was comparable to the hydrolytic activity in human saliva, and was shown to degrade dental composite and adhesives in a material-dependent manner, demonstrating clear clinical relevance for these processes as major contributors to restoration failure. However, the aforementioned study did not identify the specific esterases from the bacteria that were responsible for the hydrolysis. Therefore, the aim of this study was to identify and characterize the specific esterases with degradative activity toward methacrylate-based resin monomers from *S. mutans*, the primary cariogenic species that is associated with caries formation and progression [36]. It was hypothesized that esterases produced by *S. mutans* are stable and active in a simulated oral environment and capable of hydrolyzing dental resin monomers. The characterization of these specific esterases allows for mechanistic exploration of the biological effect of oral bacteria on dental materials, the reciprocal effect of these materials and their degradation products on the bacteria, and guide manufacturers to design materials that are more resistant to these effects. *S. mutans* UA159 was the focus of the current and previous investigations [17, 18, 35] since its genome is fully sequenced, and has relevant characteristic ‘virulence factors’ that are representative of an *S. mutans* caries-causing strain, and it is reflective of typical clinical isolates that are used *in vitro* studies with relevance to clinical conditions [37–39].

2. Materials and methods

2.1. Bioinformatic analyses

Putative esterase genes were identified by searching in the *S. mutans* UA159 genome database at The National Center for Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>). Nucleotide and deduced amino-acid sequences were analyzed using MacVector software. The signal peptide was analyzed by SignalP 4.0 server (<http://www.cbs.dtu.dk/services/SignalP/>).

2.2. Cloning, expression and purification of bacterial esterases

Basic Local Alignment Search Tool (BLAST) identified several putative esterase genes in the *S. mutans* UA159 genome database. The gene candidates (*SMU_118c*, *SMU_400*, *SMU_643*, *SMU_1443c*, *SMU_1678*) were PCR amplified from *S. mutans* UA159 genomic DNA by using designed primers (Table 1), then cloned into the p15Tv-LIC vector as previously described (empty vector was used as a control) [40], providing an N-terminal His6-tag fusion followed by a TEV protease cleavage site. The esterases were expressed in *E. coli* BL21 (DE3) then harvested for further protein isolation and purification [41]. Cells were resuspended in binding buffer [50 mM Hepes (pH 7.5), 100/300 mM NaCl, 10 mM imidazole and 2% glycerol (v/v)], lysed using a sonicator, and cell debris was removed via centrifugation at 30,000G. Cleared lysate was loaded onto a 5 mL Ni-NTA column

(QIAGEN, Dusseldorf, Germany) pre-equilibrated with binding buffer, and washed. Proteins were eluted using the above buffer with 250 mM imidazole. The His6-tag was removed by cleavage with TEV protease overnight at 4 °C in dialysis with buffer 0.3 M NaCl, 50 mM Hepes (pH 7.5), 5% glycerol and 0.5 mM tris [2-carboxyethyl] phosphine, followed by binding to Ni-NTA resin and capture of flow through. Fractions containing the proteins of interest were identified by 12.5% SDS-polyacrylamide gel electrophoresis and further purified via gel filtration on a HiLoad 16/60 Superdex75 prep-grade column [10 mM Hepes (pH 7.5) and 50 mM KCl].

2.3. Enzymatic assays

Esterase activities shown to be relevant to biodegradation of resin composites were measured using the substrates p-nitrophenyl acetate (pNPA), p-nitrophenyl butyrate (pNPB), p-nitrophenyl laurate (pNPL), o-nitrophenyl butyrate (oNPB) and butyrylthiocholine iodide (BTC) (Sigma Chemical Co. St. Louis, MO, USA) as described previously [10–13, 26–30, 35]. All experiments were performed in triplicate using a spectrophotometer (Beckman Coulter DU 800, Brea, CA) at 37°C, and corrected for the effects of non-enzymatic hydrolysis of substrates. One unit of CE-like activity was defined as the amount of enzyme producing 1 µmol p-nitrophenol from p-nitrophenyl esters per minute ($\epsilon_{401}=1.6 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) [12, 30] and specific activity was expressed as units per mg of protein. For PCE-like activity, using BTC as substrate, one unit was defined as the amount of enzyme activity producing 1 µmol butyrate per minute ($\epsilon_{405}=1.36 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$) and specific activity as units per mg of protein [12, 29, 30].

2.4. Kinetic measurements

The kinetic parameters of the purified esterase toward pNPB, a substrate that has been shown to be a good predictor for composite biodegradation [12, 29] were determined by measuring the enzyme activities with different substrate concentrations, from 0.0625 mM to 16 mM, in D-PBS (pH 7.0) at 37°C for 5 min. Kinetic parameters, the Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were calculated using the Lineweaver-Burke plot [42].

2.5. The effects of pH and resin monomers on esterase SMU_118c activity and stability

The effect of pH on enzyme activity towards pNPB was measured with the pH ranging from 3 to 7 by adding lactic acid (37°C) ($n=3/\text{pH}$), using the methods described above. The effect of resin monomers on enzyme stability was evaluated by incubating the purified enzyme (0.5 mg/mL) in 5 mL of 10 mM D-PBS (pH 7.0 and pH 5.5) with or without 100 µM bisGMA or TEGDMA at 37°C up to 21 days ($n=3$) as previously described [12]. During incubation, 0.20 mL of samples were taken at different time points (0, 1, 2, 3, 5, 14 and 21 days) for esterase activity measurement as described above.

2.6. Monomer degradation

Monomer degradation was performed in 2 mL of 10 mM PBS (pH 7.0 and pH 5.5) containing 100 µM bisGMA or TEGDMA incubated with or without 16 U/mL of purified esterase at 37°C for 125 hours ($n=3$) [12, 30]. During incubation, 0.25 mL of samples were

taken at 0, 4, 8, 24 and 125-hour time points then mixed with equal volume of pre-chilled methanol (Sigma Chemical Co. St. Louis, MO, USA) to immediately quench the reaction [11–13, 29]. For HPLC analysis, 0.4 mL of the mixed incubation solutions were filtered with centrifuge filters (UltrafreeTM-C UFC3LGC00; Millipore, Etobicoke, Ontario, Canada) to remove molecules with a molecular weight > 5000 Da in a centrifuge (H-25FI, Silencer) at 1,000 G. The processed samples were refrigerated until required for further analysis.

A high performance liquid chromatography (HPLC) system (Waters, Mississauga, ON, Canada) equipped with photodiode detector was used for the chromatographic separation and quantification of bisGMA, TEGDMA and the derived degradation products BisHPPP and MA as described previously [35]. The retention time of each product was confirmed by HPLC chromatographic profile of standard monomers and mass spectrometry (MS) analysis [11, 13, 27, 28].

2.7. Inhibition assays

The inhibitory effect of phenylmethyl sulfonyl fluoride (PMSF) (Sigma, St. Louis, MO, USA) on the enzyme was investigated by adding the inhibitor to the enzyme solution, and then measuring the enzymatic activities towards pNPB as previously described [12]. The carrier solvent of the inhibitor, 5% v/v ethanol, was assessed as a control for its effect on the enzymatic activity. PMSF and ethanol mixture was used as a non-enzyme control. The final concentration of PMSF was adjusted to 0.5 mM in the enzyme solution (16 U/mL), to provide partial inhibition as previously described [12, 43].

To further verify the reduction of enzymatic activity by PMSF, monomer degradation was conducted by incubating 100 μ M bisGMA in 3 mL of either PBS (negative control), esterase (16 U/mL), or mixture of enzyme (16 U/mL) and PMSF (0.5 mM) solution at 37°C for 72 hours (pH 7.0). A PMSF and ethanol mixture was used as a non-enzyme control. Samples from incubation solutions were analyzed by HPLC for the quantification of degradation by-product, BisHPPP.

2.8. Proteomic analysis

50 μ L of overnight cultures of *S. mutans* UA159 (N=3) were added to 3 mL of 1/4 TYEG medium (Sigma-Aldrich, St. Louis, MO, USA) in six-well polystyrene microtiter plates (Fisher Scientific, Ottawa, Ontario) and supplemented with 0.1% glucose. The cultures were incubated for 18 hours (37°C, 5% CO₂) under acidic (pH 5.5) conditions that are relevant to cariogenic conditions since the initiation of enamel demineralization and cavitation of tooth structure, as well as increased virulence of cariogenic bacteria are enhanced at this pH [17, 18, 44]. Then, the liquid contents were removed and 3 mL of phosphate-buffered saline (pH 7.4) was slowly added to each well and gently stirred to remove loosely attached cells, leaving only adhered biofilm cells. The remaining PBS was then removed and replaced with 1 mL of fresh PBS. Biofilm cells from each well were scraped and the resulting cell suspensions were transferred to 50 mL tubes and centrifuged at 2300 g for 10 minutes (4°C). The supernatants of each sample were then discarded and the biofilm cells were collected and disrupted as described previously [18]. The intracellular components were collected and separated in aliquots for the total protein concentration assessment by the Micro

Bicinchoninic Acid (Micro BCA) assay [45]. Protein contents (20 µg) were dried, denatured and reduced for 2 hours as described previously [17]. Then, peptide separation and mass spectrometric analyses were carried out as described previously [18, 45]. The obtained MS/MS spectra were searched against streptococci protein database (Swiss Prot and TrEMBL, Swiss Institute of Bioinformatics, Geneva, Switzerland, <http://ca.expasy.org/sprot/>) using SEQUEST algorithm in Proteome Discoverer 1.3 software (Thermo Scientific, San Jose, CA, USA) [17, 18, 45].

2.9. Statistical analysis

All experiments were conducted in triplicate. One-way analysis of variance (ANOVA) and Tukey's multiple comparison tests were performed to determine differences in esterase activity profiles, pH profile, enzyme stability and inhibition. Student's t-test was used to determine differences between reaction rates of esterase enzyme for degradation of bisGMA or TEGDMA at the same time-point ($p < 0.05$).

3. Results

3.1. Genes sequence analysis and expression of esterases of *S. mutans* UA159

Based on gene sequence analysis, out of ten putative esterase genes found in *S. mutans* UA159, five genes could be expressed as soluble proteins. Out of these five, three genes were successfully expressed as soluble intracellular enzymes (SMU_118c, SMU_400, and SMU_643). The empty vector had no measured activity. Activity assays confirmed that only one esterase, SMU_118c functioned as a true hydrolase toward the nitro-phenyl substrates. The deduced protein consisted of 280 amino-acid residues (Fig 1A). The N-terminal region contained no predicted secretion signal peptide.

3.2. Expression and purification of the esterase SMU_118c

SMU_118c was expressed in *E. coli* BL21 as a soluble intracellular enzyme. Homogeneity of the purified protein was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and was confirmed a molecular subunit mass of 33.8 kDa (Fig. 1B), which is similar to the molecular mass calculated from the deduced amino-acid sequence.

3.3. Substrate specificity and kinetic parameters of esterase SMU_118c

SMU_118c showed preference towards para-nitrophenyl esters, pNPA and pNPB, compared to the ortho-nitrophenyl, oNPB, ($p < 0.001$) (Fig. 2). Among the para-nitrophenyl substrates, SMU_118c showed preference towards the two-carbon acyl-chain containing pNPA rather than the four carbon acyl-chain containing pNPB ($p < 0.05$); the highest activity was 1620 U/mg towards pNPA at pH 7.0, followed by 1189 U/mg to pNPB and 80 U/mg to oNPB (Fig. 2). The enzyme had limited activity towards pNPL and BTC substrates. The K_m and V_{max} towards pNPB were 13.25 mM and 1.88 mM/min/mg protein at pH 7.0, respectively.

3.4. The effect of pH and resin monomers on the activity and stability of esterase SMU_118c

SMU_118c exhibited optimal activity toward the pNPB at pH 7.0, and the measured activity was reduced significantly with pH values lower than 6.8 ($p < 0.05$) (Fig. 3A).

After 21 days of incubation at pH 7.0, the measured activity of SMU_118c reduced to $71.8 \pm 2.4\%$ of its initial value ($p < 0.05$), and at pH 5.5, its activity reduced to $74.4 \pm 13.8\%$ of the initial value ($p > 0.05$) (Fig. 3B and 3C). The addition of bisGMA or TEGDMA did not significantly affect the activity and stability of the enzyme at both pHs ($p > 0.05$) (Fig. 3B and 3C).

3.5. Hydrolysis of the monomers by esterase SMU_118c

The amount of degraded monomers bisGMA and TEGDMA, and their by-product BisHPPP and MA, respectively, are presented as percentage of the initial molar amount of each monomer. The results were normalized to buffer control. After 125 hours, $92.5 \pm 2.5\%$ and $94.1 \pm 2.9\%$ of bisGMA was hydrolyzed by SMU_118c at pH 7.0 and 5.5 respectively (Fig. 4A). The production of BisHPPP was similar to the incremental loss of bisGMA (Fig. 4A). For TEGDMA, $17.6 \pm 4.4\%$ and $11.9 \pm 4.7\%$ of its original amount were degraded at pH 7.0 and pH 5.5 respectively (Fig. 4B). MA amounts increased to $27.6 \pm 2.2\%$ and $28.1 \pm 2.4\%$ at pH 7.0 and pH 5.5 respectively (Fig. 4B). The reaction rate of SMU_118c for bisGMA ($0.53 \pm 0.03 \mu\text{M/hr.}$) was 5.4 times higher than that of TEGDMA ($0.098 \pm 0.05 \mu\text{M/h}$) at pH 7.0, while it was 7 times higher ($0.49 \pm 0.02 \mu\text{M/hr.}$ vs. $0.07 \pm 0.03 \mu\text{M/hr.}$) at pH 5.5 ($p < 0.001$).

3.6. SMU_118c inhibition

The addition of ethanol (PMSF carrier solvent) had no significant effect on the activity of the enzyme (Fig. 5A). The addition of PMSF to SMU_118c solution decreased the relative enzymatic activity of SMU_118c to $58.3 \pm 1.2\%$ vs. the enzyme alone ($p < 0.05$). PMSF dissolved in ethanol was used as a no-enzyme control and exhibited no activity toward p-NPB.

In the monomer degradation experiments, the addition of PMSF to the buffer solutions had no effect on the hydrolysis rate of bisGMA compared to the buffer control (Fig. 5B). The addition of PMSF to SMU_118c significantly reduced the relative amount of BisHPPP production to $49.3 \pm 6.9\%$ ($p < 0.05$) (Fig. 5B).

3.7. Proteomic verification of SMU_118c expression in *S. mutans* UA159

The base-peak chromatogram for reversed-phase chromatography monitored by MS showed a consistent elution of protein/peptides in the range of 20 to 50 min, in which the esterase SMU_118c was identified from intracellular components of *S. mutans* UA159 biofilm cells.

4. Discussion

Although there are potentially several sources of hydrolytic enzymes in the oral cavity (e.g., saliva, bacteria, or leukocytes), only salivary esterases and simulated salivary esterase

activities have been extensively investigated as contributors to the degradation of the matrix component of resin composites, adhesives and the restoration-tooth interface [10–13, 20, 24–30]. More recently, it has been shown that the cariogenic bacterium, *S. mutans*, also degrades resin composites and adhesives, similarly to the degradative activities of human saliva [35]. In other bacteria, esterases have been linked to their virulence and pathogenesis [46, 47]. The above studies suggest a potential role of bacterial esterases that contribute to the degradation of the resin composite and the restoration-tooth interface, and emphasize the need to study the role of *S. mutans* esterases as a virulence factor that contributes to the progression of secondary caries [9, 10, 20, 25].

4.1 Isolation and identification of specific esterase from cariogenic bacteria

The current investigation is the first to identify and characterize an esterase, synthesized by *S. mutans* biofilms cells grown under acidic condition that hydrolyzes dental resins. With a molecular mass of 34 kDa, similar to other reported microbial esterases ranging from 20 to 60 kDa [31, 32], SMU_118C is unlikely to simply permeate the cell membrane. However, even if this enzyme is not actively secreted, it would still be available to degrade dental materials, since it has been reported that a significant portion of the cells in bacterial biofilms undergo lysis and their intracellular contents released [48]. Furthermore, the ability of whole bacteria to hydrolyze resin composites and adhesives [35] suggests that this enzyme is indeed released from the bacteria and is available to degrade dental materials and, as such, indicates that this esterase a significant virulence factor of *S. mutans*, thereby warranting further mechanistic investigation.

4.2 Activity profile and kinetic of the enzyme

Kinetic parameters of SMU_118c was assessed against pNPB, bisGMA and TEGDMA monomers as it allows for accurate characterization of the enzyme activity toward pure and well-defined substrates [12, 24]. Furthermore, the affinity toward these substrates has been previously found to be a good predictor of an enzyme's ability to degrade methacrylate-based polymers [12, 24]. Compared to other bacterial esterases, SMU_118c has a relatively high K_m toward pNPB, indicating the low affinity to such substrates. However, its high V_{max} value suggests a rapid hydrolytic rate [49], which is critical for initiating resin composite degradation and providing accessibility for salivary enzymes to the deeper layers of resin composites.

Substrate specificity of SMU_118c was determined using p-nitrophenyl-linked esters (pNPA, pNPB and pNPL) of various alkyl chain lengths (C2, C4 and C12), ortho-nitrophenyl butyrate (oNPB) with a different location of the side chain and BTC, a specific substrate for PCE-like activity assay [12, 27, 29, 30]. The enzyme was most active with p-nitrophenyl-linked esters with a short chain length (C2, C4, for pNPA and pNPB, respectively), whereas low activity was detected for substrates with longer chain lengths (C12, for pNPL), indicating that SMU_118c is a typical esterase rather than a lipase [50]. In addition, SMU_118c showed preference to substrates with chains in the para-position (pNPB) vs. those with the ortho-chains (oNPB) of the same length (C4), indicating that the enzyme activity is substrate-structure dependent. Compared to human salivary esterases that show both CE and PCE-like activity [12], SMU_118c has CE-like activity towards

nitrophenyl esters, but no PCE-like activity towards BTC. The above findings are consistent with the activity profile previously reported for the whole cell of *S. mutans* [35], suggesting that SMU_118c is a major contributor toward the hydrolase activity of this species.

4.3 Effect of pH on enzymatic activity

In the oral cavity, the pH balance is mainly influenced by cariogenic bacteria such as *S. mutans*, due to their ability to produce lactic acid (acidogenicity) by carbohydrate fermentation, reducing the local pH and initiating tooth demineralization and caries [9, 37], while also increasing virulence of the bacteria [17, 18]. Therefore, it is important to assess the ability of SMU_118c to retain its activity under relevant intra-oral pH range, adjusted by lactic acid, the end product of cariogenic bacteria. It is interesting to note that despite a reduced activity toward nitrophenyl esters at acidic pH vs. neutral pH, SMU_118c showed a similar degradative effect toward bisGMA and TEGDMA at pH 5.5 and 7.0. This apparent discrepancy could be explained by partial activity loss of SMU_118c due to protein precipitation caused by the combination of acidic pH (isoelectric precipitation) and the presence of acetonitrile (miscible solvent precipitation) in the enzyme activity assays but not in the monomer degradation assays [51]. Furthermore, compared to the p-nitrophenyl esters, the reaction sites of bisGMA and TEGDMA have distinct properties: bisGMA (pKa 31.4) and TEGDMA (pKa 32.7) exhibit weak base property at the reaction site compared to pNPA (pKa 25.0) and pNPB (pKa 26.1), so the former compounds may locally neutralize the acidic pH of the media and provide favorable pH for enzyme activity. In addition, the ester linkages in the p-nitrophenyl esters are directly linked to aromatic structure, which may cause unfavorable steric hindrance between the enzyme and the substrates after structure conformation in acidic environment, while the carbon chains linked to the ester linkage in bisGMA and TEGDMA are more flexible, allowing for steric interactions.

4.4 Stability of the enzymatic activity

Although the activity is important for the protein's biological functions, its stability is also critical for maintaining its structural integrity over time, in order to continuously carry out its function [52]. SMU_118c showed considerable stability over 21 days at both acidic and neutral pH, indicating that it could hydrolyze dental resins over a significant length of time under pathogenic growth conditions of the biofilm, potentially compromise the restoration-tooth interface, facilitating bacterial invasion and, as a result, further promoting secondary caries [9, 10, 20]. In addition to the acidic pH in the oral environment, other factors may affect enzyme activity and stability. Previous studies demonstrated that human salivary derived esterases (HSDE) were more stable than the model degradative enzyme cholesterol esterase (CE) [13, 29, 30]. However, both showed reduced activity and stability in the presence of resin-based materials or monomers [13, 29]. The current investigation demonstrated that in contrast to HSDE and CE, the presence of monomers, TEGDMA and bisGMA, had no significant effect on the activity and stability of SMU_118c at both pH 7.0 and pH 5.5 over 21 days. This suggested that the bacterial esterase maintained its ability to degrade resinous moieties for an extended period after being released, even without enzyme replenishment, thus enhancing its deleterious effect on the material and potentially on the restoration-tooth interface [13, 29, 53].

4.5 Hydrolysis of dental resins

Degradation of bisGMA was assessed by measuring the amount of its final by-product, BisHPPP in the incubation media. However, since the final byproduct of TEGDMA degradation, TEG, lacks a double bond, it cannot be identified by the PDA detector of the HPLC system used in this study. Therefore, the analyses of TEGDMA degradation was done by measuring the rate of its elimination and formation of MA in the media [12, 27]. This study demonstrated that SMU_118c was able to degrade the methacrylate-based monomers bisGMA and TEGDMA, commonly used in the production of commercial resin composite and/or adhesives [8]. The use of these well-defined monomers allowed for an accurate characterization of the enzyme's affinity toward methacrylate-based materials that was not possible with the complex whole composite material, since the latter material's multiple ingredients confound true degradative activity and preclude mechanistic exploration and generalization of the results [12, 24, 27].

SMU_118c enzymatic kinetics were specific for each monomer. bisGMA was almost completely hydrolyzed by SMU_118c after 125 hours, and corresponding amounts of its degradation product, BisHPPP, were produced during that time frame, indicating that SMU_118c catalyzed almost a complete hydrolysis of bisGMA without any measurable formation of the mono-methacrylate intermediate degradation by-product. In contrast, only $17.6 \pm 4.4\%$ and $11.9 \pm 4.7\%$ of the monomer TEGDMA were hydrolyzed at pH 7.0 and pH 5.5 respectively after 125 hours, suggesting lower affinity toward this monomer. Since, in theory, one molecule of TEGDMA monomer potentially results with the release of two MA molecules and one molecule of TEG when completely cleaved, the molar ratio of MA detectable in the HPLC system *vs.* the rate of elimination by hydrolysis of TEGDMA could provide a good estimation of the rate of degradation of the latter monomer [12, 28]. For example, $17.6 \pm 4.4\%$ (pH 7.0) of the TEGDMA original amount was degraded, and 35.2% (pH 7.0) of MA should have been theoretically produced. However, the isolated MA levels were however lower than the theoretical amount.

This imbalance of the theoretical *vs.* measured MA indicates that TEGDMA was not completely hydrolyzed by SMU_118c to form one molecule of TEG and two molecules of MA, and that the middle mono-methacrylate biodegradation product, triethyleneglycol methacrylate (TEGMA), was generated [27, 28]. This finding is in agreement with a previous study investigating resin monomer degradation by human saliva derived esterase [29]. In addition, the faster degradation of bisGMA *vs.* TEGDMA by SMU_118c, suggests that SMU_118c has higher affinity towards phenol-containing monomer than to water-soluble monomer, which is similarly to the predominant salivary esterase CE-like activity, shown to degrade both composites and adhesives [12, 30, 54]. Since bisGMA is a universal ingredient in current adhesive systems [8], the preferences of SMU_118c toward this substrate indicates high potential of the bacteria to compromise the resin dentin interface not just by the production of acid, but also by hydrolyzing a main component of the adhesive in the interface [10–13, 35].

4.6 Catalytic site of the enzyme

As CE and PCE [12, 27, 29, 30], SMU_118c belongs to the esterase-lipase superfamily with a highly conserved arrangement of serine (Ser), histidine (His) and aspartic acid (Asp) residues in the catalytic site [55, 56]. PMSF is an irreversible inhibitor of serine proteases that alkylates the hydroxyl of serine residue at the active-site [57]. PMSF's similar reduction of the hydrolysis of bisGMA and pNPB by SMU_118c suggests that the conserved active site of SMU_118c is involved in the degradation, and suggests that the hydrolysis of nitrophenyl substrates and matrix monomers occurred at the same active site. Since the active sites are highly conserved in the esterase family and the active sites of human CE are highly hydrophobic [58, 59], it is assumed the active site of SMU_118c is also hydrophobic. This property could explain the selective activity of SMU_118c towards the hydrophobic monomer bisGMA [12, 27].

4.7 Summary

To date, several studies showed degradative activities exist in human saliva [12, 24, 25, 27] and that human and simulated salivary esterases are capable of degrading resin composites and adhesives [10, 12, 27]. More recently, salivary and simulated salivary activities were shown to degrade the resin-tooth interface, comprising its integrity and increase interfacial bacterial ingress [10, 11, 13]. The current study suggests that esterase produced by the cariogenic bacterium *S. mutans* has potential to degrade and compromise the interface as well, and produce biodegradation by-products that in return, increase the virulence of the bacteria [17, 18]. Future studies should further evaluate the ability of bacterial esterases to degrade polymerized resin composites and the restoration-tooth interface, since the bacteria is a natural inhabitant in the interfacial gap that is formed by acids, human and bacterial enzymes. In addition, the biofilm structure of *S. mutans* could encapsulate esterases over the restoration's surface and within a limited volume of the resin-tooth interfacial gap, resulting in increased effective esterase concentration and resultant biodegradation by-products [10, 17, 18]. These processes could lead to a higher level of biodegradation which can compromise the interfacial seal and potentially increase in the risk of recurrent caries [10, 27] potentially reducing the longevity of the restoration [9, 20].

5. Conclusion

The esterase SMU_118c is expressed by the caries-causing bacterium *S. mutans*, under acidic conditions and can hydrolyzes methacrylate-based resin monomers, bisGMA and TEGDMA monomers, which are universally-used in resin adhesives and/or composites, while retaining its activity at acidic pH for an extended period. As such, SMU_118c could potentially contribute to the degradation of the restoration-tooth interface and acceleration of the restoration's failure rate.

Acknowledgments

The authors wish to thank Dr. A. Savchenko and Dr. D. Ma for their assistance in protein synthesis and purification, and Dr. Lida Sadeghinejad for assisting the proteomic analysis. This investigation was funded by National Institute of Dental & Craniofacial Research R01DE021385 and Canadian Institute of Health Research MOP115113. This is an independent study free of conflict of interest

References

1. Beazoglou T, Eklund S, Heffley D, Meiers J, Brown LJ, Bailit H. Economic impact of regulating the use of amalgam restorations. *Public Health Rep.* 2007; 122(5):657–63. [PubMed: 17877313]
2. Ástvaldsdóttir Á, Dagerhamn J, van Dijken JW, Naimi-Akbar A, Sandborgh-Englund G, Tranæus S, Nilsson M. Longevity of posterior resin composite restorations in adults – A systematic review. *J Dent.* 2015; 43(8):934–54. [PubMed: 26003655]
3. Opdam NJ, van de Sande FH, Bronkhorst E, Cenci MS, Bottenberg P, Pallesen U, Gaengler P, Lindberg A, Huysmans MC, van Dijken JW. Longevity of posterior composite restorations: a systematic review and meta-analysis. *J Dent Res.* 2014; 93(10):943–9. [PubMed: 25048250]
4. Murray PE, Windsor LJ, Smyth TW, Hafez AA, Cox CF. Analysis of pulpal reactions to restorative procedures, materials, pulp capping, and future therapies. *Critical Reviews in Oral Biology & Medicine.* 2002; 13(6):509. [PubMed: 12499243]
5. MJÖR IA. Clinical diagnosis of recurrent caries. *The Journal of the American Dental Association.* 2005; 136(10):1426–1433. [PubMed: 16255468]
6. Brunthaler A, König F, Lucas T, Sperr W, Schedle A. Longevity of direct resin composite restorations in posterior teeth: a review. *Clin Oral Investig.* 2003; 7(2):63–70.
7. Opdam NJ, Bronkhorst EM, Roeters JM, Loomans BA. A retrospective clinical study on longevity of posterior composite and amalgam restorations. *Dental materials.* 2007; 23(1):2–8. [PubMed: 16417916]
8. Ferracane JL. Resin composite--state of the art. *Dent Mater.* 2011; 27(1):29–38. [PubMed: 21093034]
9. Spencer P, Ye Q, Misra A, Goncalves SE, Laurence JS. Proteins, pathogens, and failure at the composite-tooth interface. *J Dent Res.* 2014; 93(12):1243–9. [PubMed: 25190266]
10. Kermanshahi S, Santerre JP, Cvitkovitch DG, Finer Y. Biodegradation of resin-dentin interfaces increases bacterial microleakage. *J Dent Res.* 2010; 89(9):996–1001. [PubMed: 20505047]
11. Serkies KB, Garcha R, Tam LE, De Souza GM, Finer Y. Matrix metalloproteinase inhibitor modulates esterase-catalyzed degradation of resin–dentin interfaces. *Dental Materials.* 2016; 32(12):1513–1523. [PubMed: 27692555]
12. Finer Y, Santerre JP. Salivary esterase activity and its association with the biodegradation of dental composites. *J Dent Res.* 2004; 83(1):22–6. [PubMed: 14691108]
13. Shokati B, Tam LE, Santerre JP, Finer Y. Effect of salivary esterase on the integrity and fracture toughness of the dentin resin interface. *Journal of Biomedical Materials Research Part B: Applied Biomaterials.* 2010; 94(1):230–237. [PubMed: 20524199]
14. Khalichi P, Cvitkovitch DG, Santerre JP. Effect of composite resin biodegradation products on oral streptococcal growth. *Biomaterials.* 2004; 25(24):5467–72. [PubMed: 15142727]
15. Khalichi P, Singh J, Cvitkovitch DG, Santerre JP. The influence of triethylene glycol derived from dental composite resins on the regulation of *Streptococcus mutans* gene expression. *Biomaterials.* 2009; 30(4):452–9. [PubMed: 18990441]
16. Stanley HR. Effects of dental restorative materials: local and systemic responses reviewed. *The Journal of the American Dental Association.* 1993; 124(10):76–80.
17. Sadeghinejad L, Cvitkovitch DG, Siqueira WL, Merritt J, Santerre JP, Finer Y. Mechanistic, genomic and proteomic study on the effects of BisGMA-derived biodegradation product on cariogenic bacteria. *Dent Mater.* 2017; 33(2):175–190. [PubMed: 27919444]
18. Sadeghinejad L, Cvitkovitch DG, Siqueira WL, Santerre JP, Finer Y. Triethylene Glycol Up-Regulates Virulence-Associated Genes and Proteins in *Streptococcus mutans*. *PLoS One.* 2016; 11(11):e0165760. [PubMed: 27820867]
19. Olea N, Pulgar R, Pérez P, Olea-Serrano F, Rivas A, Novillo-Fertrell A, Pedraza V, Soto AM, Sonnenschein C. Estrogenicity of resin-based composites and sealants used in dentistry. *Environmental Health Perspectives.* 1996; 104(3):298. [PubMed: 8919768]
20. Spencer P, Ye Q, Park J, Topp EM, Misra A, Marangos O, Wang Y, Bohaty BS, Singh V, Sene F. Adhesive/dentin interface: the weak link in the composite restoration. *Annals of biomedical engineering.* 2010; 38(6):1989–2003. [PubMed: 20195761]

21. Bornscheuer UT. Microbial carboxyl esterases: classification, properties and application in biocatalysis. *FEMS Microbiol Rev.* 2002; 26(1):73–81. [PubMed: 12007643]
22. Marashdeh M, Gitalis R, Levesque C, Finer Y. Enterococcus faecalis Hydrolyzes Dental Resin Composites and Adhesives. *Journal of Endodontics.* 2018 In press.
23. Lindqvist L, Nord C, Soder P. Origin of esterases in human whole saliva. *Enzyme.* 1976; 22(3): 166–175.
24. Cai K, Delaviz Y, Banh M, Guo Y, Santerre JP. Biodegradation of composite resin with ester linkages: identifying human salivary enzyme activity with a potential role in the esterolytic process. *Dent Mater.* 2014; 30(8):848–60. [PubMed: 24950808]
25. Delaviz Y, Finer Y, Santerre JP. Biodegradation of resin composites and adhesives by oral bacteria and saliva: a rationale for new material designs that consider the clinical environment and treatment challenges. *Dent Mater.* 2014; 30(1):16–32. [PubMed: 24113132]
26. Finer Y, Jaffer F, Santerre JP. Mutual influence of cholesterol esterase and pseudocholinesterase on the biodegradation of dental composites. *Biomaterials.* 2004; 25(10):1787–93. [PubMed: 14738842]
27. Finer Y, Santerre JP. Biodegradation of a dental composite by esterases: dependence on enzyme concentration and specificity. *J Biomater Sci Polym Ed.* 2003; 14(8):837–49. [PubMed: 14533861]
28. Finer Y, Santerre JP. The influence of resin chemistry on a dental composite's biodegradation. *J Biomed Mater Res A.* 2004; 69(2):233–46. [PubMed: 15057996]
29. Jaffer F, Finer Y, Santerre JP. Interactions between resin monomers and commercial composite resins with human saliva derived esterases. *Biomaterials.* 2002; 23(7):1707–19. [PubMed: 11922474]
30. Lin BA, Jaffer F, Duff MD, Tang YW, Santerre JP. Identifying enzyme activities within human saliva which are relevant to dental resin composite biodegradation. *Biomaterials.* 2005; 26(20): 4259–64. [PubMed: 15683649]
31. Xie G, Liu M, Zhu H, Lei B. Esterase SeE of *Streptococcus equi* ssp. *equi* is a novel nonspecific carboxylic ester hydrolase. *FEMS Microbiol Lett.* 2008; 289(2):181–6. [PubMed: 19054107]
32. Zhu H, Liu M, Sumby P, Lei B. The secreted esterase of group a streptococcus is important for invasive skin infection and dissemination in mice. *Infect Immun.* 2009; 77(12):5225–32. [PubMed: 19805529]
33. Perz V, Baumschlager A, Bleymaier K, Zitzenbacher S, Hromic A, Steinkellner G, Pairitsch A, Łyskowski A, Gruber K, Sinkel C. Hydrolysis of synthetic polyesters by *Clostridium botulinum* esterases. *Biotechnology and bioengineering.* 2016; 113(5):1024–1034. [PubMed: 26524601]
34. Perz V, Hromic A, Baumschlager A, Steinkellner G, Pavkov-Keller T, Gruber K, Bleymaier K, Zitzenbacher S, Zankel A, Mayrhofer C. An Esterase from Anaerobic *Clostridium hathewayi* Can Hydrolyze Aliphatic–Aromatic Polyesters. *Environmental science & technology.* 2016; 50(6): 2899–2907. [PubMed: 26878094]
35. Bourbia M, Ma D, Cvitkovitch DG, Santerre JP, Finer Y. Cariogenic bacteria degrade dental resin composites and adhesives. *J Dent Res.* 2013; 92(11):989–94. [PubMed: 24026951]
36. Hamada S, Koga T, Ooshima T. Virulence factors of *Streptococcus mutans* and dental caries prevention. *Journal of dental research.* 1984; 63(3):407–411. [PubMed: 6230378]
37. Ajdi D, McShan WM, McLaughlin RE, Savi G, Chang J, Carson MB, Primeaux C, Tian R, Kenton S, Jia H, Lin S, Qian Y, Li S, Zhu H, Najjar F, Lai H, White J, Roe BA, Ferretti JJ. Genome sequence of *Streptococcus mutans* UA159, a cariogenic dental pathogen. *Proc Natl Acad Sci U S A.* 2002; 99(22):14434–9. [PubMed: 12397186]
38. Burne RA, Chen YY, Wexler DL, Kuramitsu H, Bowen WH. Cariogenicity of *Streptococcus mutans* strains with defects in fructan metabolism assessed in a program-fed specific-pathogen-free rat model. *J Dent Res.* 1996; 75(8):1572–7. [PubMed: 8906125]
39. Wexler DL, Hudson MC, Burne RA. *Streptococcus mutans* fructosyltransferase (ftf) and glucosyltransferase (gtfBC) operon fusion strains in continuous culture. *Infect Immun.* 1993; 61(4):1259–67. [PubMed: 8454329]
40. Eschenfeldt WH, Lucy S, Millard CS, Joachimiak A, Mark ID. A family of LIC vectors for high-throughput cloning and purification of proteins. *Methods Mol Biol.* 2009; 498:105–15. [PubMed: 18988021]

41. Stogios PJ, Evdokimova E, Morar M, Koteva K, Wright GD, Courvalin P, Savchenko A. Structural and functional plasticity of antibiotic resistance nucleotidyltransferases revealed by molecular characterization of lincosamide nucleotidyltransferases Inu(A) and Inu(D). *J Mol Biol.* 2015; 427(12):2229–43. [PubMed: 25900373]
42. Berg, JM., Tymoczko, JL., SL. Appendix: Vmax and KM Can Be Determined by Double-Reciprocal Plots in Biochemistry. 5. New York: 2002.
43. Labow RS, Duguay DG, Santerre JP. The enzymatic hydrolysis of a synthetic biomembrane: a new substrate for cholesterol and carboxyl esterases. *J Biomater Sci Polym Ed.* 1994; 6(2):169–79. [PubMed: 7947482]
44. Matsui R, Cvitkovitch D. Acid tolerance mechanisms utilized by *Streptococcus mutans*. *Future microbiology.* 2010; 5(3):403–17. [PubMed: 20210551]
45. Siqueira WL, Bakkal M, Xiao Y, Sutton JN, Mendes FM. Quantitative proteomic analysis of the effect of fluoride on the acquired enamel pellicle. *PLoS One.* 2012; 7(8):e42204. [PubMed: 22870302]
46. Lun S, Bishai WR. Characterization of a novel cell wall-anchored protein with carboxylesterase activity required for virulence in *Mycobacterium tuberculosis*. *Journal of Biological Chemistry.* 2007; 282(25):18348–18356. [PubMed: 17428787]
47. Wall T, Båth K, Britton RA, Jonsson H, Versalovic J, Roos S. The early response to acid shock in *Lactobacillus reuteri* involves the ClpL chaperone and a putative cell wall-altering esterase. *Applied and environmental microbiology.* 2007; 73(12):3924–3935. [PubMed: 17449683]
48. Perry JA, Cvitkovitch DG, Lévesque CM. Cell death in *Streptococcus mutans* biofilms: a link between CSP and extracellular DNA. *FEMS Microbiol Lett.* 2009; 299(2):261–6. [PubMed: 19735463]
49. Sayali K, Sadichha P, Surekha S. Microbial Esterases: An overview. *Int J Curr Microbiol App Sci.* 2013; 2(7):135–146.
50. Jaeger KE, Dijkstra BW, Reetz MT. Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annu Rev Microbiol.* 1999; 53:315–51. [PubMed: 10547694]
51. Scopes, RK. Protein purification: principles and practice. Springer Science & Business Media; 2013.
52. Talley K, Alexov E. On the pH-optimum of activity and stability of proteins. *Proteins: Structure, Function, and Bioinformatics.* 2010; 78(12):2699–2706.
53. Smith RE, Burmaster S, Glaros AG, Eick JD, Walde P, Kostoryz EL, Yourtee DM. Aromatic dental monomers affect the activity of cholesterol esterase. *Biochim Biophys Acta.* 2001; 1550(1):100–6. [PubMed: 11738092]
54. Yourtee D, Smith R, Russo K, Burmaster S, Cannon J, Eick J, Kostoryz E. The stability of methacrylate biomaterials when enzyme challenged: kinetic and systematic evaluations. *Journal of biomedical materials research.* 2001; 57(4):522–531. [PubMed: 11553882]
55. Nardini M, Dijkstra BW. Alpha/beta hydrolase fold enzymes: the family keeps growing. *Curr Opin Struct Biol.* 1999; 9(6):732–7. [PubMed: 10607665]
56. Oakeshott J, Claudianos C, Campbell P, Newcomb R, Russell R. Biochemical genetics and genomics of insect esterases. *Comprehensive molecular insect science.* 2005; 5
57. Sekar V, Hageman JH. Specificity of the serine protease inhibitor, phenylmethylsulfonyl fluoride. *Biochem Biophys Res Commun.* 1979; 89(2):474–8. [PubMed: 114175]
58. Bencharit S, Edwards CC, Morton CL, Howard-Williams EL, Kuhn P, Potter PM, Redinbo MR. Multisite promiscuity in the processing of endogenous substrates by human carboxylesterase 1. *J Mol Biol.* 2006; 363(1):201–14. [PubMed: 16962139]
59. Bencharit S, Morton CL, Hyatt JL, Kuhn P, Danks MK, Potter PM, Redinbo MR. Crystal structure of human carboxylesterase 1 complexed with the Alzheimer's drug tacrine: from binding promiscuity to selective inhibition. *Chem Biol.* 2003; 10(4):341–9. [PubMed: 12725862]

Statement of Significance

The current study builds upon our highly-cited previous study by Bourbia et al., (JDR, 2013) that reported on that the cariogenic bacterium, *S. mutans* has esterase-like activities that enable the bacteria to degrade dental composites and adhesives. The current submission is the first to report on the isolation and characterization of the specific esterase activity (SMU_118c) from *S. mutans* that is a significant contributor to the whole bacterial degradative activity toward the hydrolysis of dental resins. This activity compromises the restoration-tooth interface, increase interfacial bacterial microleakage (Kermanshahi et al., JDR 2010), potentially contributing to the pathogenesis of recurrent caries around resin composite restorations. This represent a significant contribution to the field of biomaterials and their clinical performance.

Fig. 1.

(A) Nucleotide and deduced amino-acid sequences of SMU_118c (Preliminary identification of the active site is highlighted), and (B) SDS-PAGE of expression and purification of SMU_118c. An empty vector was used as a control to verify no expression of protein (not included in the final gel image).

Fig. 2.

Activity profile of SMU_118c towards different nitro-phenyl substrates at pH 7.0 and pH 5.5 (n=3; data are reported as mean $\pm$ standard errors). *represents significant differences between the two pH values for each substrate ($p < 0.05$). Values with the different upper case letters denote statistically significant differences within pH 5.5 group ($p < 0.05$). Values with the different lower case letters indicate significant differences within pH 7.0 group ($p < 0.05$).

Fig. 3.

The effect of different pH on the enzymatic activity of SMU_118c towards pNPB (A), and the relative effect of bisGMA and TEGDMA on SMU_118c activity at pH 7.0 (B) and pH 5.5 (C) following 21-day incubation (n=3; data are reported as mean $\pm$ standard errors). Values with the different lower letters indicate significant differences among different pH values ($p < 0.05$). *represents significant reduction after 21-day incubation ($p < 0.05$).

Fig. 4. SMU_118c catalyzed hydrolysis of bisGMA (A) or TEGDMA (B) at pH 7.0 and pH5.5 following up to 125-hour incubation. Results were normalized to buffer-only incubation condition (n=3; data are reported as mean $\pm$ standard errors).

Fig. 5. The inhibitory effect of PMSF (0.5 mM) on the hydrolytic activities of SMU_118c towards (A) pNPB or (B) bisGMA (n=3; data are reported as mean $\pm$ standard errors). Values with the different lower case letters indicate significant differences among groups ($p < 0.05$).

Table 1

Specific primers for PCR amplification.

Genes		Primers
SMU_118c	5' end	5'TGTATTTCAGGGCATGAAACAACATCAAAAAATAAAATATT3'
	3' end RC	5'CAAGCTTCGTCATCATGGTTCTTTCCTTAATCCCAAATAGT3'
SMU_400	5' end	5'TGTATTTCAGGGCATGACCTATCAGAAAATTATTGATAAGA3'
	3' end RC	5'CAAGCTTCGTCATCAGTCACAATTGCTATCTGAACTTTTT3'
SMU_643	5' end	5'TGTATTTCAGGGCATGAAAATTAACCTCTATGCTTTG3'
	3' end RC	5'CAAGCTTCGTCATCACTTTAAATGCAAACTCGATTAATAAAT3'
SMU_1443c	5' end	5'TGTATTTCAGGGCATGGCTTTTTTTCAGATAGAATAC3'
	3' end RC	5'CAAGCTTCGTCATCAGGACAACCGTTCCTCTTTTG3'
SMU_1678	5' end	5'TGTATTTCAGGGCATGATCATAGCAGTCTGTTTAG3'
	3' end RC	5'CAAGCTTCGTCATCAGCTATGCCAAATGCTTAACTG3'