A New Sugarcane Cystatin Strongly Binds to Dental Enamel and Reduces Erosion

A.C. Santiago[1#], M.C. Miguel[1#], Z.N. Khan[2#], V.K. Schneider[1], A. Soares-Costa[1], V.T. Pelá[2], C.C. Gironda[2], A.L. Leite[2], J.M. Edwardson[3]***, M.A.R. Buzalaf[2]**, F. Henrique-Silva[1]*

[1]Laboratory of Molecular Biology, Department of Genetics and Evolution, Federal University of São Carlos, Rodovia Washington Luis, Km 235, 13565-905, São Carlos, SP, Brazil

[2]Laboratory of Biochemistry, Department of Biological Sciences, Bauru School of Dentistry, University of São Paulo, Alameda Octávio Pinheiro Brisolla, 9-75, 17012-901, Bauru, SP, Brazil

[3]Department of Pharmacology, University of Cambridge, Tennis Court Road, Cambridge CB2 1PD, UK

[#]Authors contributed equally to this article

Corresponding authors: email: *dfhs@ufscar.br; telephone: +55 16 33518378.

**mbuzalaf@fob.usp.br; telephone: +55 14 32358346

***jme1000@cam.ac.uk; telephone: +44 1223 334014

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Abstract

Cystatin-B was recently identified as an acid-resistant protein in the acquired enamel pellicle; it could therefore be included in oral products to protect against caries and erosion. However, human recombinant cystatin is very expensive, and alternatives to its use are necessary. Phytocystatins are reversible inhibitors of cysteine peptidases that are found naturally in plants. In plants, they have several biological and physiological functions, such as regulation of endogenous processes, defense against pathogens, and response to abiotic stress. Previous studies performed by our research group have reported high inhibitory activity and potential agricultural and medical applications of several sugarcane cystatins, including CaneCPI-1, CaneCPI-2, CaneCPI-3, and CaneCPI-4. In the present study, we report the characterization of a novel sugarcane cystatin, named CaneCPI-5. This cystatin was efficiently expressed in *Escherichia coli*, and inhibitory assays demonstrated that it was a potent inhibitor of human cathepsins B, K, and L (K_i = 6.87, 0.49, and 0.34 nM, respectively). The ability of CaneCPI-5 to bind to dental enamel was evaluated using atomic force microscopy (AFM). Its capacity to protect against initial enamel erosion was also tested *in vitro*, via changes in surface hardness. CaneCPI-5 showed a very large force of interaction with enamel (compared with mucin and casein, for example) and significantly reduced initial enamel erosion. These results suggest that inclusion of CaneCPIs in dental products might confer protection against enamel erosion.

Introduction

Cystatins are competitive inhibitors of cysteine peptidases (Barrett et al. 1986; Abrahamson 1993). Different isoforms of cystatins have been identified in human saliva (Van Nieuw Amerongen et al. 2004; Fábián et al. 2012) and in the acquired enamel pellicle (AEP) (Yao et al. 2003; Vitorino et al. 2008; Siqueira et al. 2009; Delecrode et al. 2015), where they are believed to play a role in defense against microorganisms. Recently, cystatin B was identified as an acid-resistant protein in the AEP (Delecrode et al. 2015), suggesting that cystatins might be included in dental products to protect against caries and erosion. However, the cost of human recombinant cystatins is prohibitive and alternative homologs are required.

Sugarcane cystatins were first described by Reis and Margis (2001), who identified 25 cystatin-like sequences in the Sugarcane Expressed Sequence Tag (SUCEST) project database (Vettore et al. 2003). Based on their report, our research group carried out several studies involving sugarcane cystatins. CaneCPI-1 was the first sugarcane cystatin to be recombinantly expressed and characterized (Soares-Costa et al. 2002). This protein showed inhibitory activity against human cathepsins B, K, L, and V (Oliva et al. 2004). CaneCPI-2 and CaneCPI-3 were characterized by Gianotti et al. (2006) and were shown to inhibit papain; CaneCPI-3 also inhibits legumain (Santos-Silva et al. 2012). CaneCPI-4 showed inhibitory activity against human cathepsins B and L (Gianotti et al. 2008). In addition, CaneCPI-4 prevented invasion of breast cancer cells, melanoma growth, angiogenesis, and metastasis (Gianotti et al. 2008; Oliveira et al. 2011).

In this study, we report the cloning and heterologous expression of a novel sugarcane cystatin, named CaneCPI-5, and demonstrate its inhibitory activity against human cathepsins. During our studies on its ability to inhibit cysteine peptidases, it was observed that CaneCPI-5 strongly adhered to quartz cuvettes. This observation, together with the fact that its salivary homolog (cystatin B) is enriched 20 times in the AEP after exposure to citric acid under

conditions that simulate dental erosion (Delecrode et al. 2015), led us to speculate that CaneCPI-5 might bind to dental enamel and protect against initial erosion. We therefore evaluated the binding force between CaneCPI-5 and dental enamel and its ability to reduce initial enamel erosion *in vitro*, in comparison with the effects of two other proteins, mucin and casein, which have also been suggested as candidates to prevent enamel erosion (Chehaib and Lussi 2011).

Materials and Methods

Screening for the new sugarcane cystatin

Putative sequences for sugarcane cystatins were screened against the SUCEST project database using tBLASTN. Amino acid sequences of other sugarcane cystatins, including CaneCPI-1 (Soares-Costa et al. 2002), CaneCPI-2, CaneCPI-3 (Gianotti et al. 2006), and CaneCPI-4 (Gianotti et al. 2008), were used to search for similar sequences. A functional annotation of screened sequences was performed using Blas2GO (Conesa et al. 2005). Clones were selected based on the following criteria: a glycine residue in the N-terminal region, a QxVxG motif, a tryptophan residue in the C-terminal region, and the presence of the phytocystatin-specific sequence, LARFAV.

Recombinant expression of a novel cystatin in E. coli

The coding region of CaneCPI-5 from clone SCVPAM1059C07.g (GenBank: CA080618) was amplified by PCR using specific primers: CaneCPI-5-F: A**GCTAGC**CTCGCCCCCGTTCCCGG and CaneCPI-5-R: A**GGATCC**TCAGTGGGCGCGGGGCG. Restriction sites for the enzymes *Nhe*I and *Bam*HI (Thermo Fisher Scientific) (in bold) were added to the primers, which permitted insertion of the PCR product into the pET28a expression vector (Novagen) in frame with a His-tag coding

sequence. The construct was sequenced in a MegaBACE 1000 using the DYEnamic ET Dye Terminator Kit (GE Healthcare). Recombinant expression and purification of CaneCPI-5 was performed as previously described (Soares-Costa et al. 2002). Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Inhibitory activity of CaneCPI-5 against human cathepsins

The inhibitory potential of CaneCPI-5 was evaluated against human cathepsins B (Calbiochem) (1.8 nM), K (Sigma-Aldrich) (4.2 nM), and L (Calbiochem) (4.6 nM). Enzymes were pre-incubated in activity buffer (0.1 M sodium acetate buffer, pH 5.5, containing 2.5 mM DTT (USB) in a final volume of 500 µL) for 5 min at 37°C. The fluorogenic substrate Z-Phe-Arg-AMC (Calbiochem) (20 µM) was used to determine the catalytic activity of the cathepsins. CaneCPI-5 was added to the reactions at increasing concentrations, from 0.1 to 40 nM. Fluorescence changes were monitored continuously in a Hitachi F-2500 spectrofluorometer at λ_{ex}=380 and λ_{em}=460nm. The inhibitory potential of CaneCPI-5 was determined using the residual enzymatic activity of the cathepsins after addition of inhibitor. Assays were performed in triplicate. Slope values were obtained using the FL solutions 2.0 program, and the inhibition constant (K_i) was calculated following Morrison's procedure (Morrison 1982) using GraFit (Leatherbarrow 2001). Errors were less than 5% for any of the K_i values determined.

Atomic force microscopy (AFM) experiments

Enamel samples (4 x 4 x 3 mm) were prepared from sound bovine incisors. The specimens were cut from the middle of the labial aspect of the crowns using an ISOMET low-speed saw (Buehler, Lake Bluff, IL, USA) with two diamond disks (Extec, Enfield, CT, USA) separated by a 4-mm-thick spacer. The enamel surface was ground flat with water-cooled silicon-carbide discs (320-1,200 grade papers, ANSI grit; Buehler) and polished with felt paper

wetted with diamond spray. Finally, the samples were cleaned in distilled water using an ultrasonic bath to remove any residue.

Enamel samples were assigned to five groups (n=8/group): control (no protein), mucin (2.7 mg/mL), casein (10 mg/mL), mucin (2.7 mg/mL) plus casein (10 mg/mL), and CaneCPI-5 (0.85 mg/mL). For protein treatment, enamel samples were incubated in solutions containing proteins for 4 h at room temperature, with gentle agitation. After treatment, all samples were rinsed in water and dried. AFM imaging was then carried out to measure the surface roughness of the samples. All samples (control and protein-treated) were then incubated in a 0.65% citric acid solution (pH 3.5) for 1 min at room temperature with gentle agitation and imaged again. The samples were mounted on steel disks and scanned in air using a Bioscope atomic force microscope controlled by a NanoscopeIIIa controller (Bruker, Coventry, UK). The microscope was operated in tapping mode using silicon cantilevers (OTESPA-R3, Bruker, UK) with a spring constant of 26 N/m and a resonance frequency of 300 kHz. The image size was 5 µm x 5 µm. The topographic AFM images were flattened using the Nanoscope software and processed using Gwyddion 2.44 analysis software (http://gwyddion.net/). Specifically, eight diagonal profile lines (128 pixels wide) were drawn randomly on each image, to obtain the average roughness value for the image. The roughness values for the groups - control versus citric acid, control versus protein-treated, and protein-treated before and after citric acid - were compared using a paired t-test. Roughness values for the groups - citric acid-treated with and without prior protein coating - were compared using an unpaired t-test. All errors shown are standard deviations.

For single-molecule force measurement, enamel samples were mounted on steel disks in 6-cm Petri dishes and submerged in phosphate-buffered saline (pH 7.4; 4.5 ml). Mucin, casein or CaneCPI-5 were covalently coupled to silicon nitride AFM tips (the ‘C’ cantilever of MLCT probes (Bruker), which had a drive frequency of 7 kHz and a specified spring constant

of 0.01 N/m) via a flexible polyethyleneglycol (PEG18) linker. Specifically, the tips were amino-functionalized using 3-aminopropyltriethoxysilane (APTES), and then reacted with acetal-PEG18-NHS (purchased from Dr. H.J. Gruber, Johannes Kepler University, Linz, Austria). The acetal groups were converted to aldehyde groups by incubation of the tip in 1% citric acid, followed by washing in water. The aldehyde groups were then reacted with amino groups on the protein by incubation of the tip in a solution of the protein. Force measurements were performed under fluid at pH 7.4 with a Bioscope atomic force microscope (Bruker, see above). Rupture forces were obtained from force-extension curves, using appropriate software. Values for the various conditions were compared using an unpaired t-test.

Effect of protein enrichment of acquired enamel pellicle with CaneCPI-5 on initial enamel erosion

Bovine enamel samples prepared as described above were pre-selected based on initial hardness. Specifically, six indentations in the central region of the blocks were made, using a load of 50 g (Knoop indenter) for 15 s (HMV-2000; Shimadzu Corporation, Tokyo, Japan) to determine the baseline values of surface hardness (SHb). In an initial experiment, samples were assigned to five experimental groups (n=15): 1) deionized water (control), 2) 2.7 mg/mL mucin plus 5 mg/mL casein solution, 3) 0.025 mg/mL cystatin-B solution, 4) 0.025 mg/mL CaneCPI-5, and 5) 0.025 mg/mL CaneCPI-5 applied before the formation of the AEP. Three healthy volunteers donated paraffin-stimulated saliva after giving informed consent. Saliva was collected into ice-chilled vials, pooled, and centrifuged for 20 min at 4°C and 14,000 g. Except for group 5, samples were immersed in the prepared saliva for 2 h (300 µL) at 30°C with constant stirring for the formation of the AEP. They were then rinsed in deionized water and exposed to the protein solution (100 µL) for an additional 2 h at 30°C with constant stirring. For group 5, samples were first exposed to CaneCPI-5 and then the pellicle was formed by

immersion in the saliva. The samples were rinsed in deionized water and incubated in 1 mL of a 0.65% citric acid solution (pH 3.5) for 1 min at 30°C with constant stirring. They were then rinsed with deionized water and air-dried for 5 s. Each specimen was treated once/day over 3 days. The specimens were stored in a humidity chamber at 4°C between the treatments (Cheaib and Lussi 2011). Surface hardness was analysed again after days 1 and 3 (SHt) and the percentage of surface hardness change (%SHC) was calculated as a measure of enamel softening, according to the following equation: %SHC=[(SHb-SHt)/SHb]*100. Data were analysed by ANOVA and Tukey's test (P<0.05).

A second experiment was performed essentially as described above to evaluate the effect of increasing concentrations of CaneCPI-5 on the %SHC. All treatments were performed before immersion in saliva, and the erosive challenge was performed by incubation in 0.65% citric acid for 45 s instead of 1 min. Data were analysed by Kruskal-Wallis and Dunn's test (P<0.05).

Results

Sequence analysis of a novel sugarcane cystatin

Based on the sequences of CaneCPI-1, CaneCPI-2, CaneCPI-3, and CaneCPI-4, 115 cystatin sequences were selected and analyzed in Blast2GO. A clone was selected, SCVPAM1059C07.g, which encodes a cystatin that we named CaneCPI-5. The CaneCPI-5 ORF contains 411 bp and encodes a peptide of 136 amino acid residues (molecular mass approximately 14.5 kDa). The protein contains the phytocystatin conserved regions, except for the specific LARFAV sequence in the N-terminus. Alignment of CaneCPI-5 with four other sugarcane cystatins is shown in Fig. 1. Analysis using SignalP indicates a typical signal peptide, suggesting that native CaneCPI-5 is secreted.

Heterologous production of CaneCPI-5

The novel sugarcane cystatin was efficiently expressed in its soluble form. Because the protein was His-tagged, it could be purified in a single step using a Ni^{2+} affinity column. SDS-PAGE analysis revealed that CaneCPI-5 had a molecular mass of approximately 14.5 kDa, as predicted from the amino acid sequence (Fig. 2). Protein was expressed in satisfactory amounts, yielding 16 mg/L of culture.

Inhibition of cysteine peptidases by sugarcane cystatin

In general, sugarcane cystatins show good inhibitory activity against human cathepsins (Oliva et al. 2004; Gianotti et al. 2008; Oliveira et al. 2011). Consistent with this, CaneCPI-5 proved to be a potent inhibitor of cathepsins B, K, and L (K_i=6.87, 0.49, and 0.34 nM, respectively).

Effect of protein coating on surface roughness in response to acid

As shown in Fig. 3A, a typical untreated enamel sample had a relatively smooth surface with a number of linear scour lines (arrows). The mean roughness of the untreated enamel was 0.28±0.09 nm. Images of enamel that had been exposed to citric acid showed an obvious increase in surface roughness (Fig. 3B); after a 1-min treatment, the mean roughness increased to 1.06±0.15 nm (P<0.0001). Incubation of the samples with proteins (mucin, casein, mucin plus casein and CaneCPI-5) caused a visible deposition of material on the enamel surfaces, as illustrated for CaneCPI-5 in Fig. 3C; however, none of the proteins caused a statistically significant increase in mean roughness (Fig. 3D-G). In contrast, citric acid treatment caused an increase in the mean roughness of protein-coated enamel samples in all four cases. Among the individual proteins, only CaneCPI-5 significantly protected the enamel against citric acid-induced damage, although a combination of mucin plus casein also conferred protection.

Single-molecule force measurement by AFM

Typical force curves for control tips (i.e. bearing no protein) and protein-coupled tips are shown in Fig. 4. The force axes for control, mucin and casein, respectively (Fig. 4A-C), are identical to each other but different from the force axis for CaneCPI-5 (Fig. 4D). Forces detected were 0.177±0.197 nN for control tips, 0.221±0.369 nN for mucin, 0.261±0.315 nN for casein, and 0.933±0.685 nN for CaneCPI-5 (n=40). Hence, CaneCPI-5 showed a large and statistically significant (P<0.0001) force of interaction with enamel. In contrast, the forces shown by mucin and casein were not significantly different from control.

Effect of protein enrichment of AEP with CaneCPI-5 on initial enamel erosion

At day 1, treatment with cystatin B (35.1±9.9%) and with CaneCPI-5 (35.2±6.6%) before pellicle formation significantly reduced %SHC compared with control (46.9±6.7%) (Fig. 5A, P<0.05). Treatment with CaneCPI-5 (40.8±9.7%) after pellicle formation did not cause significant changes in %SHC when compared with either control or the combination mucin plus casein (45.1±13.7%). At day 3, all treatments with cystatins (54.5±8.6, 55.5±10.7 and 53.1±9.3% for cystatin B, CaneCPI-5 and CaneCPI-5 before pellicle formation, respectively) significantly reduced %SHC compared with control (67.6±9.4%) (P<0.01). In addition, treatment with CaneCPI-5 before pellicle formation significantly reduced %SHC compared with the combination mucin plus casein (64.4±9.4%) (Fig. 5B, P<0.05).

In an experiment in which the concentration of CaneCPI-5 was varied, at both time periods, only the %SHC for 0.1 and 1.0 mg/mL CaneCPI-5 significantly differed from control. However, these %SHC values did not significantly differ from each other, which indicates that 0.1 mg/mL CaneCPI-5 is probably the best concentration to be used in a rinse solution to reduce dental erosion (Fig. 5C and 5D for 1 and 3 days, respectively, P<0.05). In addition, it should

be highlighted that at day 1, the groups treated with CaneCPI-5 at 0.1 and 1.0 mg/mL showed virtually no SHC.

Discussion

In the present study, we describe the identification of a novel sugarcane cystatin and report its inhibitory activity against human cysteine peptidases. Alignment of CaneCPI-5 with CaneCPI-1, CaneCPI-2, CaneCPI-3, and CaneCPI-4 showed high similarity in the conserved domains, which are important for inhibitory activity. CaneCPI-5 showed high inhibitory activity against human cathepsins B, K, and L, which are involved in various physiological and pathological processes in the oral cavity (Dickinson 2002; Nascimento et al. 2011).

This is the first report to show the high binding force of a sugarcane cystatin (namely CaneCPI-5) to dental enamel. This result has a direct implication for the ability of this protein to protect against dental erosion by modifying the AEP, an acellular film formed on the tooth surface by selective adsorption of salivary proteins and glycoproteins (Slomiany et al. 1986). The AEP acts as a diffusion barrier or a perm-selective membrane, preventing direct contact between the acids and the tooth surface (Hannig et al. 2004). Part of the AEP is not removed from the enamel surface, even after severe erosive challenges (Hannig et al. 2009). This observation led to a recent study that identified cystatin B as an acid-resistant protein in the AEP (Delecrode et al. 2015). Cystatins then emerged as potential candidates to be included in dental products in order to prevent dental erosion. However, human recombinant cystatin is very expensive, which discourages its application in dental products. This is not the case for sugarcane cystatins. In the present study, CaneCPI-5 was shown to be an excellent alternative for modification of the AEP, conferring protection against dental erosion. Significantly, the groups treated with CaneCPI-5 performed significantly better than other proteins reported to

protect against dental erosion, such as mucin, casein and a mixture of the two (Cheaib and Lussi 2011), which can be attributed to its high binding force to dental enamel.

The experiments involving simulation of initial erosion were performed *in vitro*. In an initial experiment, specimens were exposed to saliva for 2 h to allow the formation of the AEP and then treated with solutions containing the proteins for 2 h to modify this previously formed pellicle (Chehaib and Lussi 2011). Because of the high binding force of CaneCPI-5 to enamel (as detected in the AFM experiments), some specimens were first exposed to the protein solutions (to increase their rate of binding to enamel) and the pellicle was then formed over the protein-treated enamel surface. Interestingly, the results obtained for this pre-treated group were better than those obtained for the group exposed to saliva first. It must be acknowledged that although the protection conferred by pre-treatment with CaneCPI-5 was significant when compared with control, it was modest (around 12 and 15% reduction in %SHC for days 1 and 3, respectively). This prompted us to design a concentration-response experiment, in which CaneCPI-5 was used at different concentrations and the erosive challenge was performed for a shorter period (45 s), to see if increased protection could be obtained. The degree of protection at day 1 was high and the groups treated with CaneCPI-5 at 0.1 and 1.0 mg/mL showed virtually no SHC. At day 3, however, these groups had a %SHC around 13% lower than control, indicating that the best protection occurs when CaneCPI-5 binds directly to enamel. Moreover, our results indicate that the best protective effect is achieved with solutions containing CaneCPI-5 at 0.1 mg/mL, with no additional effect at higher concentrations.

It is important to acknowledge the limitations of these *in-vitro* erosion experiments. Although the protocol is considered appropriate for preliminary investigations (Chehaib and Lussi 2011), it does not completely simulate the clinical condition. The main limitation is the time of treatment with the protein solutions (2 h). In the oral cavity, rinse solutions are often used for only 1 min. Thus, additional studies should investigate treatment with protein solutions

for more realistic times. Moreover, the model used here is static, since the same saliva remained in contact with the specimens for 2 h to form the AEP. In the clinical condition, saliva is continuously secreted and removed, which can change the pattern of protein adsorption and the degree of protection. The next natural step, then, is to evaluate the effect of solutions containing CaneCPI-5 applied for shorter periods of time to protect against initial erosion using an *in-situ* protocol. Moreover, the effect of CaneCPI-5 should be compared with those of more established agents used to prevent erosion, such as tin- and fluoride-containing solutions (Huysmans et al. 2014). Also, solutions containing CaneCPI-5 combined with tin and/or fluoride should be evaluated, as well as other vehicles for application, such as gels and toothpastes.

In conclusion, this study has identified a novel cystatin, which efficiently inhibits relevant human cysteine cathepsins, strongly binds to dental enamel and consequently protects against enamel erosion. These results open a new avenue for the inclusion of CaneCPI-5 in dental products to protect teeth against acidic injuries.

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Figure 1. Amino acid alignment of sugarcane cystatins. LARFAV and QxVxG motifs are in brackets. Black arrows indicate the glycine in the N-terminus and the tryptophan in the C-terminus. Black boxes indicate sequence identity of more than 90% and empty boxes indicate between 50% and 90% sequence identity.

Figure 2. SDS–PAGE analysis of heterologous expression, solubility, and purification of CaneCPI-5. M: BenchMark Protein Ladder (Thermo Fisher Scientific); 1: cell extract without induction; 2: cell extract after induction with IPTG; 3 and 4: insoluble and soluble fractions after cell lysis, respectively; 5: purified protein, indicated by arrow.

Figure 3. Effect of protein coating on the sensitivity of enamel to acid erosion. (A-C) Typical AFM images of a control (untreated) enamel sample (A) and samples that that had been treated with either citric acid (0.65%; B) or coated with CaneCPI-5 (C). Arrows in (A) indicate scour lines resulting from polishing. The color-height scale indicates a 0-23 nm range for (A) and (B), and a 0-25 nm range for (C). Linear scale bar, 1 µm. (D-G) Effects of coating with mucin (D), casein (E), mucin plus casein (F) or CaneCPI-5 (G), and of acid treatment without or with protein coating on enamel roughness. Note that for each protein the same enamel samples were used for the control, protein-coated and protein-coated/acid-treated groups, whereas separate samples were used for the acid-treated groups. Error bars indicate standard deviations (n=8). *, $P<0.05$; **, $P<0.001$; ***, $P<0.0001$.

Figure 4. Typical force curves. (A) Control (no protein). (B) Mucin. (C) Casein. (D) CaneCPI-5. Note the difference in force scale between (A-C) and (D).

Figure 5. Mean percentage hardness change after either 1 day (A and C) or 3 days (B and D). Enamel specimens were treated with human saliva for 2 h, then with different protein solutions for 2 h, followed by challenge with 0.65% citric acid for either 1 min (A and B) or 45 s (C and D). Treatment was performed once/day for 3 days. Error bars indicate standard deviations (n=15). For each time period, different lower-case letters denote significant differences among the groups. One-way ANOVA andTukey's test (A and B) or Kruskal-Wallis and Dunn's test (C and D) ($P<0.05$).