Optimizing the formation of the acquired enamel pellicle in vitro for proteomic analysis

Abstract

Saliva is the major contributor for the protein composition of the acquired enamel pellicle (AEP), a bacteria-free organic layer formed by the selective adsorption of salivary proteins on the surface of the enamel. However, the amount of proteins that can be recovered is even smaller under in vitro condition, due to the absence of continuous salivary flow. Objective: This study developed an in vitro AEP protocol for proteomics analysis using a new formation technique with different collection solutions. Methodology: 432 bovine enamel specimens were prepared (4x4 mm) and divided into four groups (n=108). Unstimulated saliva was provided by nine subjects. The new AEP formation technique was based on saliva resupply by a new one every 30 min within 120 minutes at 37ºC under agitation. AEP was collected using an electrode filter paper soaked in the collection solutions according with the group: 1) 3% citric acid (CA); 2) 0.5% sodium dodecyl sulfate (SDS); 3) CA followed by SDS (CA+SDS); 4) SDS followed by CA (SDS+CA). The pellicles collected were processed for analysis through LC-ESI-MS/MS technique. Results: A total of 55 proteins were identified. The total numbers of proteins identified in each group were 40, 21, 28 and 41 for the groups CA, SDS, CA+SDS and SDS+CA, respectively. Twenty-three typical AEP proteins were identified in all groups, but Mucin was only found in CA and CA+SDS, while three types of PRP were not found in the SDS group. Moreover, a typical enamel protein, Enamelin, was identified in the CA+SDS group only. Conclusion: The new technique of the in vitro AEP formation through saliva replacement was essential for a higher number of the proteins identified. In addition, considering practicality, quantity and quality of identified proteins, citric acid seems to be the best solution to be used for collection of AEP proteins.

Keywords: Pellicle. Enamel. Saliva. Proteomics. Methods.
Introduction

Saliva is formed mainly by the secretion of salivary glands. This fluid is essential for the homeostasis of the oral cavity, since it cleans, lubricates and protects the oral tissues, as well as acting as a buffering agent and source of calcium and phosphate ions for remineralization of the teeth. Moreover, saliva is the major contributor for the protein composition of the acquired enamel pellicle (AEP), a bacteria-free organic layer formed by the selective adsorption of salivary proteins on the surface of the enamel, but containing also carbohydrates, neutral lipids, phospholipids and glycolipids. These organic components grant important functions to the AEP that acts as a diffusion barrier, reducing the direct contact of the acids with the tooth surface, slowing down tooth dissolution.

The ability of the AEP to protect the enamel surface against acids is due mainly to its protein composition, especially by the proteins present in the basal layer. These remain in the AEP after exposure to acids and are currently objects of great interest, since they might protect against dental caries and erosion. In the last few years, proteomic approaches have been used to identify these proteins so that they can be added to dental products which, when applied, could modify the composition of the AEP, increasing its protective potential against acids.

One of the main difficulties faced in the studies involving proteomic analysis of the AEP is the small amount of proteins that can be obtained, which can impair analysis, both in vitro, in situ and in vivo. The amount of proteins that can be recovered is even smaller under in vitro condition, due to the absence of continuous salivary flow. Moreover, in the in vivo studies available so far, AEP samples collected from 8-10 volunteers are pooled in order to obtain enough proteins to be analyzed by mass spectrometry, which does not allow proper assessment of the biological variation among the volunteers in future studies.

Methodology

Ethical aspects and subjects

This study was approved by the local Ethics Committees (Human and Animal, protocols 86772718.0.0000.5417 and 007/2018, respectively) of Bauru School of Dentistry, University of São Paulo, SP, Brazil).

Nine young adult subjects of both genders took part in the study, after signing an informed consent document. The exclusion criteria for the volunteers were: presence of caries lesions, use of medication that could change the salivary flow, gingivitis, smoking habit, periodontitis, low salivary flow (unstimulated and stimulated flows should be greater than 0.1 and 1.0 mL/minute, respectively).

The volunteers received a kit containing a toothbrush, toothpaste and floss for oral hygiene standardization. In the morning (to avoid circadian effects), after oral hygiene (2 hours), unstimulated saliva was collected from each volunteer in tubes, kept in ice. Saliva samples were immediately centrifuged (4.500 x g at 4°C, 15 min). The supernatants were collected, pooled and added to a 1:100 protease inhibitor (phenylmethane sulfonyl fluoride - PMSF, N-Ethyhlmaleimide - NEM and Phenantroline). Saliva supernatants were stored at -80ºC, until use.

Preparation of bovine specimens

Bovine incisors underwent a process of screening and cleaning (removal of soft tissue) before
preparation. Each tooth was glued on an acrylic plate with thermoactive dental plaster (Kerr Corporation, Orange, CA, EUA) for the separation of the root and coronary portions. The crowns were cut using a precision cutting machine (ISOMET Low Speed Saw Buehler Ltd., Lake Bluff, IL, EUA), with two diamond discs (double-sided XL 12205 high concentration; 102 × 12.7 × 0.3 mm³; Extec Diamont Wafering Blade, Enfield, CT, USA) separated by a 4-mm thick spacer, in order to obtain 4 × 4 × 2 mm enamel specimens.

Study groups

A total of 432 standardized bovine enamel specimens were obtained and divided into four groups (n=108/group), according to the solution used to collect the AEP, as follows: 1) 3% citric acid (CA); 2) 0.5% sodium lauryl sulfate (SDS); 3) CA followed by SDS (CA+SDS); 4) SDS followed by CA (SDS+CA).

Formation of AEP in vitro

For the formation of the AEP, the specimens were placed in 96-well microplates in which 250 µL of saliva were added. The AEP was then allowed to form for 120 min. For the constant control of the temperature and agitation, a ThermoMixer® (Eppendorf ThermoMixer® C, Hamburg, Germany) was used at 37°C, under agitation. The mainly particularity in this study was the new methodology adopted regarding the resupply of saliva. For this, during the AEP formation (120 min), saliva was exchanged three times (every 30 min). This way, the previous saliva was removed and a new sample was immediately added (250 µL).

Collection of the AEP

After the formation of AEP, the specimens were immediately withdrawn from saliva and washed with a small spray of deionized water for three seconds and air dried. The AEP was collected using an electrode filter paper 5 × 10 mm (Electrode Wick, Bio-Rad, Hercules, CA, USA) soaked in the collection solutions according with the respective group. The excess of the acid was removed with absorbent paper. For CA+SDS and SDS+CA groups, one filter paper was used for the first solution and a new filter paper was used for the second one. One filter paper was used for 6 specimens only and then resupplied by a new one.

For AEP collection, each paper soaked with their respective solution was rubbed (no pressure) on the enamel surface, with the aid of tweezers. The filter papers used to collect AEP from the specimens of the same group were placed in 2 mL tubes and stored at -80°C. The experiment was repeated for additional 2 consecutive days.

Shotgun proteomics analysis by NanoLC-ESI-MS/MS

The methods were exactly as described elsewhere. The papers with the samples were cut into small pieces with the aid of sterile scissors and tweezers. The filter papers containing the AEP collected from 3 different days (triplicate collection) for each of the groups were pooled to obtain enough amount of AEP proteins to be submitted to the proteomic analysis.

The peptides identification was performed on a nanoACQUITY UPLC-Xevo QTof MS system (Waters, Manchester, UK). In addition, ProteinLynx Global Server (PLGS) version 3.0 was used to process and search the continuum LC-MSE data. Samples from each group were analyzed in triplicate (technical triplicates). Proteins were searched for on the Homo sapiens proteome database (reviewed only, UniProtKB/Swiss-Prot) downloaded on April 2017 from UniProtKB (http://www.uniprot.org/). Finally, the identified proteins were classified and assigned by biological function, origin and molecular interaction (http://www.uniprot.org/) (Table S1).

Results

The total amount of AEP proteins recovered was very similar for all the groups, ranging between 26 and 33 µg. A total of 55 proteins were identified (Figure 1), among which are 20 proteins typically found in the AEP, such as two isoforms of Alpha-amylase, two isoforms of Basic salivary proline-rich protein, three isoforms of Cystatin, five isoforms of Hemoglobin, Lysozyme, Mucin-7, Pancreatic alpha-amylase, Proline-rich protein 4, Protein S100-A9, Salivary acidic proline-rich phosphoprotein ½, Statherin and Submaxillary gland androgen-regulated protein 3B (Table S1).

The total numbers of proteins identified in each group were 40, 21, 28 and 41 for CA, SDS, CA+SDS and SDS+CA, respectively. Among them, 15, 14, 14 and 9 are proteins typically found in the AEP (Table 1). Additionally, the proteins found exclusively in one of the groups was 8, 0, 5 e 4 for the groups CA, SDS, CA+SDS and SDS+CA, respectively (Table 1; Figure 1).
Fifteen proteins were identified in all groups (Figure 1), most of them being proteins typically described in the AEP, such as Pancreatic alpha-amylase, Submaxillary gland androgen-regulated protein 3B, Immunoglobulin heavy constant alpha 1, Immunoglobulin heavy constant alpha 2, two isoforms of Alpha-amylase, three isoforms of Cystatin, Lysozyme C and Statherin (Table S1).

Remarkably, Mucin-7 was only identified in the CA and CA+SDS groups, while Protein S100-A9 was only found in the CA and SDS+CA groups. On the other hand, isoforms of Hemoglobin were only detected in the SDS and SDS+CA groups. Moreover, a typical enamel protein, Enamelin, was identified in the CA+SDS group only. Furthermore, 3 types of PRP were not found in the SDS group (Table S1).

Discussion

The proteomic analysis of AEP formed in vitro is an important tool in pre-clinical studies since it allows preliminary evaluation of preventive agents for dental caries and dental erosion. In addition, in in vitro studies it is possible to recover the enamel specimens over which the AEP is formed to be submitted to distinct tests, which is not feasible in vivo. However, to date there is only one study where the proteomic profile of the AEP formed in vitro was evaluated. In this sense, our main aim was to develop an in vitro protocol of the AEP formation using different solutions previously described in the literature to collect AEP proteins for shotgun proteomic analysis.

The main reason for such scarcity of studies is the small amount of proteins that can be recovered from the in vitro formed AEP, whereas that in in vivo condition the AEP is formed under continuous salivary flow, which is not present in vitro. In order to overcome this, in this study we resupplied the saliva in which the specimens were immersed every 30 min during the two-hour period of AEP formation. This procedure was successful for an in vitro study, since it allowed recovery of approximately 30 µg of proteins that is enough for proper proteomic analysis. In contrast, pilot studies performed for the definition of this protocol with the absence of saliva exchange demonstrated the failure in the recovery proteins of the AEP (data not shown). Despite the fact that saliva was resupplied every 30 to increase the total amount of recovered proteins, it is possible that the solution used to collect the AEP proteins may also influence the amount of recovered proteins.

To date, most of the studies available in the literature employ 3% citric acid for collected of the acquired pellicle. However, in these studies, the proteins collected from 8-10 volunteers are pooled in order to obtain enough amount of proteins to be analyzed by mass spectrometry, i.e., it is not possible to perform individual analysis. More recently, the pellicle proteins formed on ceramic specimens in situ were eluted by incubation in TRIS-HCl buffer containing SDS, followed by ultrasonication in RIPA-buffer. This procedure allowed analysis of individual samples with high inter-individual and inter-day consistency. However, it cannot be done in vivo, due to the necessity of sonication and to the toxicity of the detergents employed. SDS has been employed...
| Accession number | Protein Name                          | Score  | CA | SLS | CA + SLS | SLS + CA |
|------------------|---------------------------------------|--------|----|-----|----------|----------|
| P68032           | Actin_ alpha cardiac muscle 1 (d, m, n, q, u, w) | 65.6055 | X | x  | x        | x        |
| P68133           | Actin_ alpha skeletal muscle (b, d, m, n, q, u, w) | 65.6055 | x | x  | x        | x        |
| P62736           | Actin_ aortic smooth muscle (b, d, m, n, q, u) | 65.6055 | x | x  | x        | x        |
| P60709           | Actin_ cytoplasmic 1 (b, m, n, q, u, w) | 65.6055 | x | x  | x        | x        |
| P63251           | Actin_ cytoplasmic 2 (a, d, g, j, n, q, u, w) | 65.6055 | x | x  | x        | x        |
| P63287           | Actin_ gamma-entric smooth muscle (b, m, n, q, u, w) | 65.6055 | x | x  | x        | x        |
| P04745           | Alpha-amylose 1 (a, g, o, u) | 452.4455 | x | x  | x        | x        |
| P19961           | Alpha-amylose 2B (a, g, o, u) | 579.3912 | x | x  | x        | x        |
| G5397X6          | Basic salivary proline-rich protein 1 (b, l, o, u) | 155.8623 | x | x  | x        | x        |
| P02812           | Basic salivary proline-rich protein 2 (b, l, o, u) | 155.8623 | x | x  | x        | x        |
| Q562R1           | Beta-actin-like protein 2 (b, m, n, u, w) | 78.1257 | x | x  | x        | x        |
| Q66RL1           | BRCA1-A complex subunit RAP80 (d, m, p, u) | 47.3122 | x | x  | x        | x        |
| P33856           | Breast cancer type 1 susceptibility protein (b, e, i, j, o, u) | 85.8217 | x | x  | x        | x        |
| Q8N4G4           | CA6 protein (a, m, u) | 76.7328 | x | x  | x        | x        |
| P23280           | Carbonic anhydrase 6 (a, g, o, u) | 301.6657 | x | x  | x        | x        |
| Q98XL7           | Caspase recruitment domain-containing protein 11(c, e, m, n, s, w) | 60.8389 | x | x  | x        | x        |
| P08603           | Complement factor H (b, m, o, w) | 38.0628 | x | x  | x        | x        |
| Q02851           | Complement factor H-related protein 1 (a, m, o, w) | 38.0628 | x | x  | x        | x        |
| P01036           | Cystatin-S (a, b, g, o, u) | 2640.733 | x | x  | x        | x        |
| P09228           | Cystatin-S (a, b, g, o, u) | 2646.624 | x | x  | x        | x        |
| P01037           | Cystatin-SN (a, b, g, o, u) | 2646.624 | x | x  | x        | x        |
| Q9UGM3           | Deleted in malignant brain tumors 1 protein (f, m, n, o, v, w) | 86.0094 | x | x  | x        | x        |
| E3 SUMO-protein ligase PIAS2 (e, m, p, u) | 24.2121 | x | x  | x        | x        |
| Q8NRM1           | Enamelin (b, d, m, o, w) | 15.9628 | x | x  | x        | x        |
| P66707           | Hemoglobin subunit beta (b, c, m, n, o, w) | 293.9594 | x | x  | x        | x        |
| P02042           | Hemoglobin subunit delta (b, c, m, n, o, w) | 293.9594 | x | x  | x        | x        |
| P02100           | Hemoglobin subunit epsilon (b, c, m, n, u) | 293.9594 | x | x  | x        | x        |
| P69892           | Hemoglobin subunit gamma-2 (b, c, m, n, u) | 293.9594 | x | x  | x        | x        |
| P01876           | Immunoglobulin heavy constant alpha 1 (b, e, i, j, o, u) | 866.7542 | x | x  | x        | x        |
| P01877           | Immunoglobulin heavy constant alpha 2 (b, e, i, j, o, u) | 806.5165 | x | x  | x        | x        |
| P15919           | Immunoglobulin J chain (b, m, o, w) | 946.0537 | x | x  | x        | x        |
| Q8WYH8           | Inhibitor of growth protein 5 (b, m, p, u) | 95.4649 | x | x  | x        | x        |
| Q9H1B7           | Interferon regulatory factor 2-binding protein-like (b, m, p, u) | 9.3029 | x | x  | x        | x        |
| P31025           | Lipoalpin-1 (a, b, m, o, w) | 623.5907 | x | x  | x        | x        |
| P07162           | Lysozyme C (a, b, g, i, j, o, u, w) | 268.2844 | x | x  | x        | x        |
| Q8TA7X           | Mucin-7 (b, i, k, o, u) | 417.1399 | x | x  | x        | x        |
| C8JT7N           | Nucleolin T/A-1 isofrom p40 (b,m,n,x) | 92.8821 | x | x  | x        | x        |
| Q04746           | Pancreatic alpha-amylose (a, g, o, u) | 1996.417 | x | x  | x        | x        |
| Q8583J           | POTE ankyrin domain family member E (b, m, o, u) | 65.6055 | x | x  | x        | x        |
| A5A3E0           | POTE ankyrin domain family member F (b, m, o, u) | 65.6055 | x | x  | x        | x        |
| P02814           | Submaxillary gland androgen-regulated protein 3B (a, g, o, u, w) | 3959.276 | x | x  | x        | x        |
| P17987           | T-complex protein 1 subunit alpha (e, m, n, u, w) | 59.5312 | x | x  | x        | x        |
| Q8G6S9           | Uncharacterized protein (m, t, x) | 420.9096 | x | x  | x        | x        |
| A0A087WZY1       | Zinc-alpha-2-glycoprotein (a, b, g, o, u, w) | 496.7979 | x | x  | x        | x        |

Classification of proteins according to: General Function: a) metabolism; b) biological process; c) transport; d) structure and structural organization; e) information pathways; f) miscellaneous; Function in AP: g) metabolism; h) tissue regeneration; i) antimicrobial; j) immune response; k) lubrication; l) biomineralization; m) unknown biological function; Origin: n) cytoplasm origin; o) extracellular origin; p) nucleus origin; q) cytoskeleton origin; r) intracellular origin; s) membrane origin; t) unknown protein origin; Interaction: u) protein/protein interaction; v) calcium/phosphate binding; w) other molecular interaction; x) unknown molecular interaction. The groups are: 3% citric acid (CA), 0.5% sodium lauryl sulfate (SLS), 3% citric acid plus 0.5% sodium lauryl sulfate (CA+SLS) and 0.5% Sodium lauryl sulfate plus 3% citric acid (SLS+C).
### Table 1 - Proteins identified in the acquired enamel pellicle formed *in vitro* on enamel specimens and collected using different solutions

| Group | Accession number | Protein Name | Score |
|-------|-----------------|--------------|-------|
| CA    | P68032          | Actin_ alpha cardiac muscle 1 | 65.6055 |
|       | P68133          | Actin_ alpha skeletal muscle | 65.6055 |
|       | P62736          | Actin_ aortic smooth muscle | 65.6055 |
|       | P60709          | Actin_ cytoplasmic 1 | 65.6055 |
|       | P63261          | Actin_ cytoplasmic 2 | 65.6055 |
|       | P63267          | Actin_ gamma-enteric smooth muscle | 65.6055 |
|       | P04745          | Alpha-amylase 1 | 452.4455 |
|       | P19961          | Alpha-amylase 2B | 579.3912 |
|       | G5E0X6          | Basic salivary proline-rich protein 1 | 155.8623 |
|       | P02812          | Basic salivary proline-rich protein 2 | 155.8623 |
|       | Q502R1          | Beta-actin-like protein 2 | 78.1257 |
|       | Q96RL1*         | BRCA1-A complex subunit RAP80 | 47.3122 |
|       | P36958*         | Breast cancer type 1 susceptibility protein | 85.8217 |
|       | P23280          | Carbonic anhydrase 6 | 144.9005 |
|       | Q88X7*          | Caspase recruitment domain-containing protein 11 | 60.8839 |
|       | P01036          | Cystatin-S | 2640.733 |
|       | P09228          | Cystatin-SA | 451.4857 |
|       | P101037         | Cystatin-SN | 2646.624 |
|       | Q9UGM3          | Deleted in malignant brain tumors 1 protein | 86.0094 |
|       | P01876          | Immunoglobulin heavy constant alpha 1 | 866.7542 |
|       | P01877          | Immunoglobulin heavy constant alpha 2 | 806.5165 |
|       | P31025          | Lipocalin-1 | 623.5907 |
|       | P61626          | Lysozyme C | 268.2844 |
|       | Q8TAX7          | Mucin-7 | 417.1399 |
|       | C9JTN*          | Nucleolysin TIA-1 isform p40 | 92.8821 |
|       | P04746          | Pancreatic alpha-amylase | 1996.417 |
|       | Q668J3          | POTE ankyrin domain family member E | 65.6055 |
|       | A5A3E0          | POTE ankyrin domain family member F | 65.6055 |
|       | A0A0A0MT31      | Proline-rich protein 4 | 420.9096 |
|       | P06702          | Protein S100-A9 | 711.9667 |
|       | Q8BYX7          | Putative beta-actin-like protein 3 | 65.6055 |
|       | QEQSPM4         | Putative lipocalin 1-like protein 1 | 204.5444 |
|       | P02810          | Salivary acidic proline-rich phosphoprotein 1/2 | 420.9096 |
|       | Q8NB9*          | Sodium-coupled neutral amino acid transporter 9 | 255.0823 |
|       | Q8WB9*          | Sodium-independent sulfate anion transporter | 262.1345 |
|       | P02808          | Statherin | 54090.52 |
|       | P02814          | Submaxillary gland androgen-regulated protein 3B | 3959.276 |
|       | P17987*         | T-complex protein 1 subunit alpha | 59.5312 |
|       | A9A087WZY1      | Uncharacterized protein | 420.9096 |
| SDS   | P25311*         | Zinc-alpha-2-glycoprotein | 496.7979 |
|       | P04745          | Alpha-amylase 1 | 274.6967 |
|       | P19961          | Alpha-amylase 2B | 274.6967 |
|       | Q8NA4G4         | CA6 protein | 76.7328 |
|       | P23280          | Carbonic anhydrase 6 | 301.6657 |
|       | P01036          | Cystatin-S | 293.6917 |
|       | P09228          | Cystatin-SA | 216.0704 |
|       | P01037          | Cystatin-SN | 274.2227 |
|       | Q9UGM3          | Deleted in malignant brain tumors 1 protein | 56.2783 |
|       | P68871          | Hemoglobin subunit beta | 293.9594 |
|       | P02042          | Hemoglobin subunit delta | 293.9594 |
|       | P02100          | Hemoglobin subunit epsilon | 293.9594 |
|       | P69891          | Hemoglobin subunit gamma-1 | 293.9594 |
|       | P69892          | Hemoglobin subunit gamma-2 | 293.9594 |
|       | P01876          | Immunoglobulin heavy constant alpha 1 | 290.1472 |
|       | P01877          | Immunoglobulin heavy constant alpha 2 | 9.2098 |
|       | P31025          | Lipocalin-1 | 1070.104 |

Continued on the next page
| P61626 | Lysozyme C | 731.9259 |
| P04746 | Pancreatic alpha-amylase | 36.6661 |
| Q5VSP4 | Putative lipocalin 1-like protein 1 | 1070.104 |
| P02808 | Statherin | 20250.94 |
| P02814 | Submaxillary gland androgen-regulated protein 3B | 1497.902 |
| CA+SDS | P04745 | Alpha-amylase 1 | 181.0646 |
| P19961 | Alpha-amylase 2B | 166.898 |
| G5E9X6 | Basic salivary proline-rich protein 1 | 552.4909 |
| P02812 | Basic salivary proline-rich protein 2 | 552.4909 |
| Q8N4G4 | CA6 protein | 67.728 |
| Q8N4G4 | CA6 protein | 47.9429 |
| P23280 | Carbonic anhydrase 6 | 728.2514 |
| P08603* | Complement factor H | 38.0628 |
| Q03591* | Complement factor H-related protein 1 | 38.0628 |
| P01036 | Cystatin-S | 1556.063 |
| P09228 | Cystatin-SA | 1013.174 |
| P01037 | Cystatin-SN | 205.9523 |
| Q9UGM3 | Deleted in malignant brain tumors 1 protein | 118.9028 |
| OT592B* | E3 SUMO-protein ligase Pias2 | 24.2121 |
| Q9NRM1* | Enamelin | 15.9628 |
| P01876 | Immunoglobulin heavy constant alpha 1 | 154.7424 |
| P01877 | Immunoglobulin heavy constant alpha 2 | 79.6826 |
| Q8WYH8* | Inhibitor of growth protein 5 | 95.4849 |
| P31025 | Lipocalin-1 | 1275.895 |
| P61626 | Lysozyme C | 1199.526 |
| Q8TAX7 | Mucin-7 | 93.5897 |
| P04746 | Pancreatic alpha-amylase | 166.898 |
| A0A0A0MT31 | Proline-rich protein 4 | 325.6618 |
| Q5VSP4 |Putative lipocalin 1-like protein 1 | 1275.895 |
| P02810 | Salivary acidic proline-rich phosphoprotein 1/2 | 325.6618 |
| P02808 | Statherin | 32088.14 |
| P02814 | Submaxillary gland androgen-regulated protein 3B | 1053.619 |
| A0A087WZY1 | Uncharacterized protein | 325.6618 |
| SDS+CA | P68032 | Actin_alpha cardiac muscle 1 | 145.4612 |
| P68133 | Actin_alpha skeletal muscle | 145.4612 |
| P62736 | Actin_aortic smooth muscle | 145.4612 |
| P60709 | Actin_cytoplasmic 1 | 145.4612 |
| P63201 | Actin_cytoplasmic 2 | 145.4612 |
| P63267 | Actin_gamma-enteric smooth muscle | 145.4612 |
| P04745 | Alpha-amylase 1 | 3352.857 |
| P19961 | Alpha-amylase 2B | 3008.341 |
| G5E9X6 | Basic salivary proline-rich protein 1 | 174.755 |
| P02812 | Basic salivary proline-rich protein 2 | 174.755 |
| Q562R1 | Beta-actin-like protein 2 | 72.3325 |
| Q8N4G4 | CA6 protein | 48.5939 |
| P23280 | Carbonic anhydrase 6 | 836.3896 |
| P01036 | Cystatin-S | 1501.204 |
| P09228 | Cystatin-SA | 657.3894 |
| P01037 | Cystatin-SN | 1529.473 |
| Q9UGM3 | Deleted in malignant brain tumors 1 protein | 233.5314 |
| P68871 | Hemoglobin subunit beta | 761.2395 |
| P02042 | Hemoglobin subunit delta | 761.2395 |
| P02100 | Hemoglobin subunit epsilon | 761.2395 |
| P69892 | Hemoglobin subunit gamma-2 | 761.2395 |
| P01876 | Immunoglobulin heavy constant alpha 1 | 621.9611 |
| P01877 | Immunoglobulin heavy constant alpha 2 | 182.5975 |
to collection AEP proteins in vivo in order to perform immunoblotting analysis. Since SDS is biocompatible and can be used to collect AEP proteins in vivo, in the present study we evaluated both 3% citric acid and 0.5% SDS, alone or in combination, in order to develop a method of collection of AEP proteins that results in large amount of proteins and can be employed in different protocols (in vitro, in situ and in vivo).

The obtained results indicate that the amount of proteins (ranging between 26 and 33 µg) recovered when these solutions were used was satisfactory, especially considering an in vitro study. Moreover, among the 55 proteins identified in all groups, 15 are common to all of them, most of which are classical players of the AEP. It could be expected that the combinations CA + SDS or SDS + CA could increase the total number of identified proteins, in comparison to CA or SDS only, since the acid and the detergent could be expected to remove different proteins of the AEP. However, this was not the case, since the total number of identified proteins were 40, 21, 28 and 41 for CA, SDS, CA + SDS and SDS + CA groups, respectively. It is also important to consider the quality of proteins recovered when 3% citric acid was used is satisfactory, especially considering the in vitro protocol of this study. Moreover, the amount of proteins recovered when CA was used (around 30 µg) might be enough to allow proteomic analysis of biological triplicates, since not assessing the biological variability is currently the major shortcoming of the proteomic studies of the AEP. It would be desirable to compare the proteomic profile of AEPs formed in vitro, in situ and in vivo, so that the results of in vitro and in situ studies can be extrapolated to the clinical condition.

Thus, the results obtained indicate that the new technique develop by resupply of saliva for the AEP formation in the present study was essential for a higher number of the proteins identified by proteomics analysis. In addition, 3% citric acid is, among the tested solutions, the best one to remove AEP proteins for shotgun proteomic analysis. The amounts and quality of proteins recovered when 3% citric acid was used is satisfactory, especially considering the in vitro protocol of this study. Moreover, the amount of proteins recovered when CA was used (around 30 µg) might be enough to allow proteomic analysis of biological triplicates, since not assessing the biological variability is currently the major shortcoming of the proteomic studies of the AEP. It would be desirable to compare the proteomic profile of AEPs formed in vitro, in situ and in vivo, so that the results of in vitro and in situ studies can be extrapolated to the clinical condition.

Acknowledgments

The authors thank FAPESP for the scholarships to the first (2017/04857-4) and second (2017/05031-2) authors. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

Authors' contributions

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* Proteins exclusively identified in each group. Proteins highlighted in bold are typical of the acquired enamel pellicle. The groups are: 3% citric acid (CA), 0.5% sodium dodecyl sulfate (SDS), 3% citric acid plus 0.5% sodium dodecyl sulfate (CA+SDS) and 0.5% Sodium dodecyl sulfate plus 3% citric acid (SDS+CA).

| P01591* | Immunoglobulin J chain | 946.0537 |
| Q9H1B7* | Interferon regulatory factor 2-binding protein-like | 9.3029 |
| P31025 | Lipocalin-1 | 1312.528 |
| P61626 | Lysozyme C | 4389.076 |
| P04746 | Pancreatic alpha-amylase | 3061.443 |
| Q6S5J3 | POTE ankyrin domain family member E | 117.0785 |
| A5A3E0 | POTE ankyrin domain family member F | 117.0785 |
| P0CG38* | POTE ankyrin domain family member I | 69.466 |
| P0CG39* | POTE ankyrin domain family member J | 69.466 |
| A0A0A0MT31 | Proline-rich protein 4 | 368.7032 |
| P06702 | Protein S100-A9 | 132.0728 |
| Q9BYX7 | Putative beta-actin-like protein 3 | 47.6124 |
| Q5VSP4 | Putative lipocalin 1-like protein 1 | 1295.604 |
| P02810 | Salivary acidic proline-rich phosphoprotein 1/2 | 368.7032 |
| P02808 | Statherin | 32670.96 |
| P02814 | Submaxillary gland androgen-regulated protein 3B | 1295.599 |
| A0A087WZY1 | Uncharacterized protein | 368.7032 |

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