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STABILITY OF LACTATE DEHYDROGENASE, ASPARTATE AMINOTRANSFERASE, ALKALINE PHOSPHATASE AND TARTRATE RESISTANT ACID PHOSPHATASE IN HUMAN SALIVA AND GINGIVAL CREVICULAR FLUID IN THE PRESENCE OF PROTEASE INHIBITOR

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Abstract - The stability of aspartate aminotransferase (AST), lactate dehydrogenase (LDH), tartrate resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) activities from saliva and gingival crevicular fluid (GCF) with and without the addition of protease inhibitor (PI) at room temperature (RT; 25°C), 4°C and -20°C were investigated. AST, LDH, TRAP and ALP activities in saliva and GCF (n=9) with and without the addition of PI were assayed at 0 (control), 12, 24, 48, 72 h, one and two weeks. A paired t-test showed there were a significant differences ($p<0.05$) between LDH and TRAP activities in saliva, in the presence and without PI at all temperatures. ALP activity exhibited a significant difference in activity ($p<0.05$) in the presence and without PI at RT while no significant differences were observed at 4°C and -20°C. A significant difference ($p<0.05$) was observed in AST, LDH and TRAP activities (GCF) at RT and 4°C in the presence and without PI. We conclude that PI is essential for maintaining stable enzyme activities in saliva and GCF.

Key words: Protease inhibitor, saliva, gingival crevicular fluid

INTRODUCTION

A biomarker is defined as an indicator of normal biological, pathogenic processes, or pharmacological responses to a therapeutic or other health care intervention (Atkinson et al., 2001). Several constituents of saliva and gingival crevicular fluid (GCF) have been characterized as biomarkers, notably several enzymes that are proving to be very useful in screening, diagnosis and tooth movement study (Nomura et al., 2012). As certain enzymes in saliva and GCF have short half-lives, they need to be analysed immediately after collection. However, there are others enzymes that remain stable after longer periods after collection, even at room temperature (~25° C) (Koh and Koh, 2007). Limitations in enzymological analysis are logistic and financial, and collection and preparation of sample procedures has made immediate sample analysis impossible. Unstable temperature and/or bacterial contamination due to complex procedures affect the enzymological activities of saliva and GCF samples during sampling and storage (Rohaya et al., 2010). Therefore, an optimized storage procedure prior to sample analysis is necessary.

The major enzyme constituents of saliva were identified as biomarkers for diagnostic purposes (Koh and Koh, 2007), and salivary analysis is becoming an important tool in detecting physiological and pathological changes (Amerongen et al., 2004). Saliva is an oral fluid found in the oral cavity that is secreted by major and minor salivary glands. It is 99% water and contains electrolytes (sodium, potassium, calcium, chloride, magnesium) and proteins (Patricia et al., 2008; Lima et al., 2010). Human salivary glands secrete approximately 500 – 1500 mL/day saliva in response to sympathetic and parasympathetic stimulations (Chiappin et al., 2007; Pfaffe et al., 2011). Saliva also contains non-salivary constituents derived from gingival crevicular fluid, expectorated bronchial secretions, serum, blood cells from oral wounds, bacteria and bacterial products, fungi and viruses, desquamated epithelial cells and food debris (Nomura et al., 2012). Therefore, saliva plays an important role in oral health and homeostasis (Chiappin et al., 2007).

GCF is an exudate that flows into the oral cavity from the periodontal pocket (Lamster and Ahlo, 2007). It is produced as a result of reaction between bacterial biofilm adherent to the tooth surface and the cell of the periodontal tissue (Pradeep et al., 2009). Many of the components of GCF, such as cytokines, interleukins, enzymes and prostaglandins have been characterized as biomarkers. These biomarkers serve as diagnostic or prognostic markers of periodontal disease, oral health and healing after therapy (Subrahmanyam and Sangeetha, 2003; Pradeep et al., 2009). GCF is composed of substances derived from serum, leukocytes, structural cells of the periodontium and oral bacteria. The content of GCF reflects immune reactions and host-parasite interactions (Gastel et al., 2011).

Lactate dehydrogenase (LDH), aspartate aminotransferase (AST), tartrate resistant acid phosphatase (TRAP) and alkaline phosphatase (ALP) in human saliva and GCF have the potential to serve as biological marker for orthodontic tooth movement (OTM) monitoring (Rohaya et al., 2008; Rohaya et al., 2009; Rohaya et al., 2011). AST is a cytoplasmic enzyme present in many cells (Paolantonio et al., 2000). Cell damage and cell death releases AST into extracellular fluid and can be readily assayed in the oral cavity, in both GCF and saliva. LDH is a cytoplasmic enzyme which catalyses the reversible oxidation reaction of lactate to pyruvate (Numabe et al., 2004). After cell death, LDH is released into the extracellular environment. Hence, LDH activity levels exhibit positive correlation with tissue inflammation during gingivitis and tissue destruction caused by periodontitis in human (Serra et al., 2003). ALP is an enzyme related to tissue mineralization that is synthesized and released by dental pulp cells such as fibroblasts and odontoblast (Perinetti et al., 2004). ALP can also be used as biochemical marker to determine osteoblast activity since it is present on the fragments of plasma membranes of osteoblast cells (Huang et al., 2010). TRAP is a metallophosphoesterase known to play a role in iron transport, generation of reactive oxygen species, osteopontin phosphatase and is a differentiation factor of hematopoietic and osteoblastic cells. TRAP is expressed by osteoclasts, bone-resorbing cells, and certain subpopulations of monocytes/macrophages and dendritic cells (Ljusberg, 2005). Bone resorption during orthodonthic treatment is accompanied by elevated acid phosphatase activities in GCF and saliva (Insoft, 1996).

In this study, the stability of LDH, AST, TRAP and ALP activities from human saliva and GCF samples in the presence and without the addition of protease inhibitor (PI) at three different temperatures (room temperature (25 ^{0}C), 4 °C and -20 °C) after 12 h, 24 h, 48 h, 72 h, one week and two weeks were investigated. The results of this study will provide information about the suitability of temperature at different storage periods and the addition of protease inhibitor to preserve enzyme activity in saliva and GCF samples.

MATERIALS AND METHODS

Sample collection

This study was conducted at School of Biosciences and Biotechnology and Orthodontic Postgradu-

Table 1. Percentage of aspartate aminotransferase (AST) in the presence and absence of protease inhibitor in saliva at room temperature, 4^{0}C and -20^{0}C for various time of storage (n=9).

Time	Percentage of AST activity with PI (%)			Percentage of AST activity without PI (%)			Percentage of AST activity with PI vs Percentage of AST activity without PI (%)		
	RT	4^{0}C	-20^{0}C	RT	4^{0}C	-20^{0}C	RT	4^{0}C	-20^{0}C
0 h	100.0±0.4	100.0±0.4	100.0±0.4	100.0±0.4	100.0±0.4	100.0±0.4	♦♦	♦♦	♦♦
12 h	94.0±0.2	102.0±0.9	121.0±2.0	151.0±1.9	122.0±1.2	96.0±1.8	♦♦	♦♦	♦♦
24 h	100.0±0.7	108.0±0.8	110.0±0.4	129.0±1.3	121.0±1.2	122.0±1.3	♦♦	♦♦	♦♦
48 h	**80.0±0.6•**	**94.0±0.6•**	101.0±0.4	101.0±1.1	99.0±1.2	103.0±1.0	♦♦	♦♦	♦♦
72 h	**104.0±0.5***	**115.0±0.4***	109.0±0.3	131.0±1.3	105.0±1.2	108.0±1.1	♦♦	♦♦	♦♦
1 week	**92.0±0.4•**	**83.0±0.4•**	82.0±0.2	99.0±0.7	113.0±1.9	89.0±0.7	♦♦	♦♦	♦♦
2 week	**126.0±0.7***	**132.0±0.5***	**136.0±0.5***	**163.0±1.2***	111.0±0.9	**130.0±1.4***	♦♦	♦♦	♦♦

Data represent the percentage (%) of AST activity ± SEM for 0, 12, 24, 48, 72 h, 1 and 2 weeks (0 h is the control) at three different temperature; RT, 4^{0}C and -20^{0}C. * = significant increase (bold), •= significant decrease (italic, bold), ♦ = significant, ♦♦ = not significant.

Table 2. Percentage of aspartate aminotransferase (AST) in the presence and absence of protease inhibitor in GCF at room temperature, 4^{0}C and -20^{0}C for various time of storage (n=9).

Time	Percentage of AST activity with PI (%)			Percentage of AST activity without PI (%)			Percentage of AST activity with PI vs Percentage of AST activity without PI (%)		
	RT	4^{0}C	-20^{0}C	RT	4^{0}C	-20^{0}C	RT	4^{0}C	-20^{0}C
0 h	100.0±0.2	100.0±0.2	100.0±0.2	100.0±0.2	100.0±0.2	100.0±0.2	♦♦	♦♦	♦♦
12 h	109.0±0.3	117.0±0.4	114.0±0.4	135.0±0.4	124.0±0.3	124.0±0.2	♦♦	♦♦	♦♦
24 h	**283.0±0.5***	**290.0±0.5***	**165.0±0.5***	**326.0±0.7***	**292.0±0.8***	**306.0±0.7***	♦♦	♦♦	♦♦
48 h	**260.0±0.7***	**266.0±0.3***	**154.0±0.7***	**268.0±0.2***	**252.0±0.6***	**240.0±0.8***	♦♦	♦♦	♦♦
72 h	**284.0±0.9***	**308.0±0.3***	**161.0±0.5***	**302.0±0.2***	**242.0±0.3***	**250.0±0.5***	♦♦	♦♦	♦♦
1 week	86.0±0.2	92.0±0.3	87.0±0.5	75.0±0.1	80.0±0.1	84.0±0.1	♦♦	♦♦	♦♦
2 week	104.0±0.1	104.0±0.3	84.0±0.3	**133.0±0.4***	138.0±0.5	119.0±0.2	♦	♦	♦♦

Data represent the percentage (%) of AST activity ± SEM for 0, 12, 24, 48, 72 hour, 1 and 2 weeks, at three different temperature; RT, 4^{0}C and -20^{0}C. * = significant increase (bold), •= significant decrease (italic, bold), ♦ = significant, ♦♦ = not significant

ate Clinic, Department of Orthodontic, Faculty of Dentistry, Universiti Kebangsaan Malaysia. Saliva and GCF samples were collected from nine healthy subjects (n=9) that fulfilled the following criteria: no systemic illness, non-smokers, no use of any form of inflammatory drugs before the beginning of the study and good oral hygiene and periodontal health.

Saliva collection

Saliva was collected in a sterile 15 mL centrifuge tube. Before saliva collection, subjects were told not to perform any oral activities for 90 minutes in order to collect unstimulated saliva. Subjects were asked to rinse their mouth with sterile water prior to spitting. After collection, the tube was centrifuged

Table 3. Percentage of lactate dehydrogenase (LDH) in the presence and absence of protease inhibitor in saliva at room temperature, 4⁰C and -20⁰C for various time of storage (n=9).

Time	Percentage of LDH activity with PI (%)			Percentage of LDH activity without PI (%)			Percentage of LDH activity with PI vs Percentage of LDH activity without PI (%)		
	RT	4⁰C	-20⁰C	RT	4⁰C	-20⁰C	RT	4⁰C	-20⁰C
0 h	100.0±0.3	100.0±0.3	100.0±0.3	100.0±0.3	100.0±0.3	100.0±0.3	♦♦	♦♦	♦♦
12 h	71.0±0.8	101.0±0.5	95.0±0.7	80.0±0.5	95.0±0.7	95.0±0.7	♦♦	♦♦	♦♦
24 h	89.0±0.7	87.0±0.5	95.0±0.6	85.0±0.7	85.0±0.6	85.0±0.7	♦♦	♦♦	♦♦
48 h	79.0±0.6	84.0±0.7	90.0±0.6	91.0±0.7	93.0±0.7	87.0±0.6	♦♦	♦♦	♦♦
72 h	96.0±0.8	99.0±0.7	98.0±0.6	100.0±0.7	95.0±0.6	99.0±0.7	♦♦	♦♦	♦♦
1 week	**81.0±0.4**•	88.0±0.7	90.0±0.6	**123.0±0.2***	**75.0±0.5**•	88.0±0.6	♦	♦	♦♦
2 week	**186.0±0.8***	93.0±0.5	136.0±1.9	**133.0±0.1***	**72.0±0.5**•	**79.0±0.5**•	♦	♦	♦

Data represent the percentage (%) of LDH activity ± SEM for 0, 12, 24, 48, 72 hour, 1 and 2 weeks at three different temperature; RT, 4⁰C and -20⁰C. * = significant increase (bold), •= significant decrease (italic, bold), ♦ = significant, ♦♦ = not significant

Table 4. Percentage of lactate dehydrogenase (LDH) in the presence and absence of protease inhibitor in GCF at room temperature, 4⁰C and -20⁰C for various time of storage (n=9)

Time	Percentage of LDH activity with PI (%)			Percentage of LDH activity without PI (%)			Percentage of LDH activity with PI vs Percentage of LDH activity without PI (%)		
	RT	4⁰C	-20⁰C	RT	4⁰C	-20⁰C	RT	4⁰C	-20⁰C
0 h	100.0±0.3	100.0±0.3	100±0.33	100.0±0.3	100.0±0.3	100.0±0.3	♦♦	♦♦	♦♦
12 h	89.0±0.2	96.0±0.1	91±0.19	92.0±0.1	91.0±0.2	89.0±0.1	♦♦	♦♦	♦♦
24 h	95.0±0.2	93.0±0.2	102±0.11	131.0±1.3	94.0±0.3	92.0±0.2	♦♦	♦♦	♦♦
48 h	89.0±0.3	94.0±0.2	83±0.14	87.0±0.2	91.0±0.1	91.0±0.2	♦♦	♦♦	♦♦
72 h	114.0±0.1	105.0±0.4	100±0.65	139.0±0.7	96.0±0.6	98.0±0.2	♦♦	♦♦	♦♦
1 week	**85.0±0.2**•	87.0±0.1	**84±0.16**•	**83.0±0.2**•	**58.0±0.3**•	**84.0±0.3**•	♦	♦	♦♦
2 week	**84.0±0.2**•	**51.0±0.8**•	**90±0.25**•	**84.0±0.2**•	**70.0±0.1**•	**77.0±0.5**•	♦♦	♦♦	♦♦

Data represent the percentage (%) of LDH activity ± SEM for 0, 12, 24, 48, 72 hour, 1 and 2 week, at three different temperature; RT, 4⁰C and -20⁰C. * = significant increase (bold), •= significant decrease (italic, bold), ♦ = significant, ♦♦ = not significant

for 10 min at 1000 xg (4 ⁰C). The supernatant was transferred into a new centrifuge tube and enzyme activities was determined.

Gingival crevicular fluid (GCF) collection

GCF was collected using periopaper (Proflow, USA) from the gingival sulcus of canine teeth. Each periopaper strip was inserted 1-2 mm into the gingival crevice of test teeth and was left *in situ* for 60 s. Then, a total of three periopapers from each subject were placed into a 1.5 mL microcentrifuge tube containing 1280 µL bovine serum albumin (0.01 mg/mL). The tube was centrifuged for 10 min at 400 xg (4⁰C), in a microcentrifuge (Hettich Zentrifugen Mikro 22R) to elute the GCF component.

Table 5. Percentage of tartrate resistant acid phosphatase (TRAP) in the presence and absence of protease inhibitor in saliva at room temperature, 4^0C and -20^0C for various time of storage (n=9)

Time	Percentage of TRAP activity with PI (%)			Percentage of TRAP activity without PI (%)			Percentage of TRAP activity with PI vs Percentage of TRAP activity without PI (%)		
	RT	4^0C	-20^0C	RT	4^0C	-20^0C	RT	4^0C	-20^0C
0 h	100.0±0.5	100.0±0.5	100.0±0.5	100.0±0.5	100.0±0.5	100.0±0.5	♦♦	♦♦	♦♦
12 h	97.0±0.1	101.0±0.1	117.0±3.5	103.0±1.3	86.0±0.3	94.0±1.6	♦♦	♦♦	♦♦
24 h	99.0±0.1	91.0±1.5	92.0±2.9	99.0±2.2	85.0±1.1	83.0±2.1	♦♦	♦♦	♦♦
48 h	**52.0±0.3·**	**57.0±0.8·**	87.0±1.0	**48.0±0.4·**	**60.0±0.8·**	66.0±2.9	♦♦	♦♦	♦♦
72 h	**87.0±0.4·**	**83.0±0.9·**	81.0±1.6	**68.0±1.1·**	**73.0±0.7·**	80.0±0.8	♦♦	♦♦	♦♦
1 week	**72.0±0.8·**	**55.0±0.3·**	39.0±0.9	**72.0±0.2·**	**75.0±0.5·**	**72.0±0.5·**	♦♦	♦	♦
2 week	**67.0±0.5·**	**75.0±2.7·**	**38.0±0.2·**	**56.0±0.3·**	**43.0±0.6·**	**38.0±0.1·**	♦	♦	♦♦

Data represent the percentage (%) of TRAP activity ± SEM for 0, 12, 24, 48, 72 hour, 1 and 2 weeks, at three different temperature; RT, 4^0C and -20^0C. * = significant increase (bold), ·= significant decrease (italic, bold), ♦ = significant, ♦♦ = not significant

Table 6. Percentage of tartrate resistant acid phosphatase (TRAP) in the presence and absence of protease inhibitor in GCF at room temperature, 4^0C and -20^0C for various time of storage (n=9)

Time	Percentage of TRAP activity with PI (%)			Percentage of TRAP activity without PI (%)			Percentage of TRAP activity with PI vs Percentage of TRAP activity without PI (%)		
	RT	4^0C	-20^0C	RT	4^0C	-20^0C	RT	4^0C	$-20\ ^0$C
0 h	100.0±0.2	100.0±0.2	100.0±0.2	100.0±0.2	100.0±0.2	100.0±0.2	♦♦	♦♦	♦♦
12 h	83.0±0.1	82.0±0.1	87.0±0.1	101.0±0.2	85.0±0.1	79.0±0.1	♦♦	♦♦	♦♦
24 h	76.0±0.1	74.0±0.1	71.0±0.1	138.0±1.9	52.0±0.2	84.0±0.1	♦♦	♦♦	♦♦
48 h	53.0±0.2	80.0±0.2	76.0±0.1	127.0±0.2	**39.0±0.1·**	67.0±0.1	♦♦	♦♦	♦♦
72 h	**44.0±0.1·**	**34.0±0.1·**	78.0±0.1	76.0±0.1	**49.0±0.3·**	59.0±0.2	♦	♦♦	♦♦
1 week	**61.0±0.1·**	**66.0±0.1·**	**70.0±0.1·**	**65.0±0.1·**	**57.0±0.1·**	**63.0±0.1·**	♦♦	♦♦	♦♦
2 week	**86.0±0.1·**	**84.0±0.1·**	**85.0±0.1·**	126.0±0.7	**84.0±0.1·**	**80.0±0.1·**	♦	♦♦	♦♦

Data represent the percentage (%) of TRAP activity ± SEM for 0, 12, 24, 48, 72 hour, 1 and 2 weeks, at three different temperature; RT, 4^0C and -20^0C. * = significant increase (bold), ·= significant decrease (italic, bold), ♦ = significant, ♦♦ = not significant

Sample storage

Both saliva and GCF samples were divided equally into two ~700 μL. Protease inhibitor (GE Healthcare) was added to one of the samples at a ratio of 1:100 and subsequently divided into 18 microcentrifuge tubes (0.5 mL). The samples with and without protease inhibitor were stored at room temperatures (RT), 4°C, and -20°C. ALP, TRAP, LDH and AST activities were measured after 0 h, 12 h, 24 h, 48 h, 72 h, 1 and 2 weeks of storage. Measurements of ALP, TRAP, LDH and AST activities were performed in triplicate.

Enzyme assay

Alkaline phosphatase (ALP) and tartrate resistant acid phosphatase assay (TRAP)

ALP activities in saliva and GCF samples were measured spectrophotometrically using microplate

Table 7. Percentage of alkaline phosphatase (ALP) in the presence and absence of protease inhibitor in saliva at room temperature, 4ºC and -20ºC for various time of storage (n=9)

Time	Percentage of ALP activity with PI (%)			Percentage of ALP activity without PI (%)			Percentage of ALP activity with PI vs Percentage of ALP activity without PI (%)		
	RT	4ºC	-20ºC	RT	4ºC	-20ºC	RT	4ºC	-20ºC
0 h	100.0±0.1	100.0±0.1	100.0±0.1	100.0±0.1	100.0±0.1	100.0±0.1	♦♦	♦♦	♦♦
12 h	58.0±0.1	73.0±0.1	92.0±0.3	122.0±0.5	130.0±0.1	132.0±0.3	♦♦	♦♦	♦♦
24 h	92.0±0.1	96.0±0.2	100.0±0.5	157.0±0.5	115.0±0.2	89.0±0.20	♦♦	♦♦	♦♦
48 h	71.0±0.1	101.0±0.3	124.0±0.7	140.0±0.1	106.0±0.3	113.0±0.27	♦	♦♦	♦♦
72 h	75.0±0.1	100.0±0.3	105.0±0.5	**167.0±0.1***	132.0±0.3	131.0±0.20	♦	♦♦	♦♦
1 week	83.0±0.1	75.0±0.1	102.0±0.8	**167.0±0.1***	122.0±0.1	105.0±0.16	♦	♦♦	♦♦
2 week	82.0±0.2	86.0±0.2	94.0±0.7	**179.0±0.2***	148.0±0.2	129.0±0.28	♦	♦♦	♦♦

Data represent the percentage (%) of ALP activity ± SEM for 0, 12, 24, 48, 72 hour, 1 and 2 weeks at three different temperature; RT, 4ºC and -20ºC. * = significant increase (bold), •= significant decrease (italic, bold), ♦ = significant, ♦♦ = not significant

Table 8. Percentage of alkaline phosphatase (ALP) in the presence and absence of protease inhibitor in GCF at room temperature, 4ºC and -20ºC for various time of storage (n=9)

Time	Percentage of ALP activity with PI (%)			Percentage of ALP activity without PI (%)			Percentage of ALP activity with PI vs Percentage of ALP activity without PI (%)		
	RT	4ºC	-20ºC	RT	4ºC	-20ºC	RT	4ºC	-20ºC
0 h	100.0±1.0	100.0±1.0	100.0±1.0	100.0±1.0	100.0±1.0	100.0±1.0	♦♦	♦♦	♦♦
12 h	75.0±0.2	105.0±0.3	108.0±0.3	80.0±0.6	95.0±0.9	103.0±0.7	♦♦	♦♦	♦♦
24 h	101.0±0.2	88.0±0.3	104.0±0.2	95.0±0.8	89.0±0.8	86.0±0.4	♦♦	♦♦	♦♦
48 h	162.0±0.6	151.0±0.4	130.0±0.3	**132.0±0.9***	**111.0±0.4***	99.0±0.8	♦♦	♦♦	♦♦
72 h	144.0±0.7	163.0±0.5	150.0±0.4	**111.0±0.9***	**124.0±0.7***	125.0±0.8	♦♦	♦♦	♦♦
1 week	**169.0±0.1***	**188.0±0.5***	**174.0±0.5***	**127.0±0.9***	**138.0±1.0***	**141.0±1.1***	♦♦	♦♦	♦♦
2 week	**216.0±0.4***	**244.0±0.7***	**208.0±0.9***	**162.0±0.7***	**146.0±0.5***	**157.0±1.1***	♦♦	♦♦	♦♦

Data represent the percentage (%) of ALP activity ± SEM for 0, 12, 24, 48, 72 hour, 1 and 2 weeks, at three different temperature; RT, 4ºC and -20ºC. * = significant increase (bold), •= significant decrease (italic, bold), ♦ = significant, ♦♦ = not significant

reader (Varioskan, Thermo Fisher Scientific) at 405 nm. The samples were added into a mixture containing *p*-nitrophenyl phosphate (1mM), mannitol (20mM), magnesium chloride (0.3 mM) and carbonate buffer (pH 9.8), and were incubated at 30°C for 30 min.

For TRAP activity, saliva and GCF samples were incubated at 37 °C for 60 min in a mixture containing *p*-nitrophenyl phosphate (10 mM), ascorbic acid (1 mM), $FeCl_3$ (0.1 mM), potassium chloride (0.15 M), sodium tartrate (10 mM), sodium acetate buffer pH 5.8 (0.1 M) and 0.1% (v/v) Triton X-100. Absorbance was determined at 405 nm. A standard curve employing 1 mM of *p*-nitrophenol solution was used to convert absorbance into enzyme unit activity (1 U = 1 μmol of *p*-nitrophenol released per minute at 37°C).

Lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) assay

LDH and AST activities were determined spectrophotometrically at 340 nm, using a microplate reader (Varioskan, Thermo Fisher Scientific). For LDH, samples were added into a test tube containing 16.2 mM sodium pyruvate, 0.54 M phosphate buffer (pH 7.4) and 0.2 mM NADH after 5 min incubation at 30^{0}C. Changes in the absorbance was recorded every 30 s for 3 minutes.

For AST activity, samples were assayed for 4 min in a mixture of 0.15 M L-aspartate, 0.1 mM 2-oxoglutarate, 0.2 mM NADH, 0.4 U malate dehydrogenase and 0.1 M sodium phosphate buffer (pH 7.4) after 5 min incubation at 30^{0}C. One unit of enzyme activity = 1 mol NADH consumed per min at 30 ^{0}C. Specific activities of LDH and AST are expressed in units of activity (U) per mg protein.

Data analysis

All the specific activity results were converted into percentage; 0 hour was considered as 100 % activity. The data obtained were analysed with paired t-test, using Statistical Package for Social Sciences (SPSS) ver. 20 software (SPSS Inc., USA); $p<0.05$ was considered as significant.

RESULTS

Aspartate aminotransferase (AST) activity in saliva and GCF samples

Significant increases ($p<0.05$) were observed for salivary AST activity in the presence of PI (for 72 h at RT and 4^{0}C; 2 weeks at RT, 4^{0}C and -20^{0}C) and also without PI in saliva after storage for 2 weeks at RT and -20 ^{0}C (Table 1). A significant decrease in AST activity ($p<0.05$) was observed after the addition of PI in saliva stored for 48 h and 1 week at RT and 4^{0}C (Table 1). AST activity significantly increased ($p<0.05$) with and without the addition of PI in GCF, stored for 24 h and 72 h at RT, 4^{0}C and -20^{0}C, and for 2 weeks at RT without PI (Table 2).

Lactate dehydrogenase (LDH) activity in saliva and GCF samples

LDH activity increased significantly ($p<0.05$) in the presence of PI after 2 weeks at RT (Table 3), and without PI, in saliva stored for 1 and 2 weeks at RT (Table 3). A significant decrease in LDH activity was observed in the presence of PI in samples stored for 1 week at RT (Table 3). Without addition of PI a significant decrease ($p<0.05$) was observed in samples kept for 1 and 2 weeks at 4^{0}C (Table 3). There were no significant increase ($p>0.05$) in LDH activity neither with nor without addition of PI in GCF (Table 4). In GCF, LDH activity significantly decreased with and without the addition of PI after 1 and 2 weeks of storage at all temperatures, except at 4^{0}C at 1 week with PI (Table 4).

Tartrate resistant acid phosphatase (TRAP) activity in saliva and GCF samples

There were no significant increases ($p>0.05$) in TRAP activity with and without the addition of PI in saliva and GCF stored at all examined temperatures (Table 5 and 6). The addition of PI to the saliva caused a significant decrease in enzyme activity after 48 h, 72 h, 1 and 2 weeks at RT and 4^{0}C, and after 2 weeks at -20 ^{0}C (Table 5). Significant decreases ($p<0.05$) in TRAP activity were observed without the addition of PI to saliva, starting from 48 h at all investigated temperatures except at -20^{0}C where the decrement started after 1 week of storage (Table 5). In the GCF, the activity of TRAP decreased significantly ($p<0.05$) with the addition of PI, starting from 72 h at RT and 4 ^{0}C, whereas at -20 ^{0}C it decreased after 1 week. Without the addition of PI, significant decrease ($p<0.05$) was observed in samples kept at RT for 1 week at 4°C and starts from 48 h and -20 °C starts from 1 week (Table 6).

Alkaline phosphatase (ALP) activity in saliva and GCF samples

No significant decreases ($p>0.05$) were observed for ALP activity in saliva stored in the presence of PI for all investigated parameters (Table 7). However, a

significant increase ($p<0.05$) was observed for ALP activity in the absence of PI, starting from 72 h at RT (Table 7). In GCF there was not a significant decrease ($p>0.05$) in ALP activity after addition of PI for all investigated parameters. A significant increase ($p<0.05$) in ALP activity was observed with the addition of PI, starting from the 1st week at all temperatures (Table 8), and without the addition of PI at RT and at 4^{0}C, starting from 48 h and at -20^{0}C starting from the 1st week of storage.

Enzymatic activity in saliva and GCF

The significant changes in AST, LDH, TRAP and ALP activities with and without addition of PI in saliva and GCF storage at various temperatures (RT, 4 ^{0}C and -20 ^{0}C) were also analysed. There was no significant difference ($p>0.05$) of AST activity in saliva (Table 1). The analysis shows significant differences ($p<0.05$) in LDH activity (at RT and 4^{0}C for 1 week and 2 weeks, -20^{0}C for week 2; Table 3), TRAP activity (at RT for 2 week, at 4^{0}C for 1 and 2 weeks and -20^{0}C for 1 week; Table 5) and ALP activity (at RT starts from 48 h; Table 7) in saliva.

There were significant differences ($p<0.05$) for AST activity (at RT and 4^{0}C after 2 weeks (Table 2), LDH activity at RT and 4 ^{0}C at 1 week (Table 4) and TRAP activity at RT, 72 h and 2 weeks (Table 6) in GCF. ALP activity showed no significant difference ($p>0.05$) (Table 8).

DISCUSSION

Study on the stability of enzymes in saliva and GCF samples in the presence protease inhibitor at various temperatures can provide information as to optimal sample management.

Rohaya et al. (2010) examined the stability of human salivary LDH activity in three different storage media (EDTA, glycerol, and PEG) at room temperature (RT), 4^{0}C, and -20^{0}C for one and two weeks. In contrast with our study, protease inhibitor (PI) was added to saliva and GCF samples at three different temperature (RT, 4^{0}C, and -20^{0}C) for certain times of storage (12 h, 24 h, 48 h, 72 h, 1 week and 2 week). The present study observed the stability of enzymes that are involved in tooth movement processes - AST, LDH, TRAP and ALP.

In saliva, AST activity did not show significant difference ($p<0.05$) between addition and without addition of PI (Table 1). The data suggests that PI is not important for maintaining AST activity. In contrast, LDH (Table 3) and TRAP (Table 5) activities showed significant differences ($p<0.05$) between in the presence and absence of PI when stored for more than 1 week at the three temperatures. ALP activity displayed significant difference ($p<0.05$) in the presence and absence of PI at RT, while at 4^{0}C and -20^{0}C a significant differences ($p>0.05$) was not observed (Table 7). Therefore, PI is needed when saliva is stored for more than 1 week at RT, 4^{0}C and -20^{0}C to maintain LDH and TRAP activity, and at RT to maintain ALP activity.

For GCF, there were significant differences ($p<0.05$) in the presence and absence of PI for a storage more than 72 h for AST, LDH and TRAP at RT and AST and LDH at 4^{0}C. At -20^{0}C, all the enzymes did not show any significant difference ($p>0.05$) in the presence and absence of PI. Therefore, PI is not necessary for the GCF sample to be stored at -20^{0}C (Tables 2, 4 and 6). However, the temperature of the storage should be taken into consideration since different temperatures can stabilize enzyme activities for different times of storage, either with or without addition of PI (Tables 1 – 8; Koh and Koh, 2007). It is well known that storage at RT often causes protein degradation and that enzymes can be deactivated by microbial growth (Rohaya et al., 2010). In addition, some enzymes will lose their activity when frozen due to changes in their structures (Katherine et al., 2000). The addition of PI is required for longer samples storage, i.e. saliva and GCF especially at RT (Table 2-3; Table 5-7).

Previous study on stability of salivary LDH with EDTA, glycerol and PEG by Rohaya et al. (2010), showed that PEG as the best storage media for salivary LDH, whereas, EDTA only suitable at room

temperature for one week time and glycerol only suitable in the cold. Moreover, they also reported that salivary LDH activity without addition of any chemicals was significantly decreased ($p<0.01$) after a week. This finding was similar with current research where salivary LDH activity decreased with time after storage at 4^0C and -20^0C. A study by De La Pena et al. (2004) showed that salivary LDH can be stored up to 3 month at 4^0C without addition of any stabilizer or inhibitor. At -20^0C, they found a significant decrease ($p<0.05$) in LDH activity after only 30 min of storage.

In the study by Halleen (2006), TRAP activity was stabilized up to 2 days at RT, 3 days at 4^0C and 1 month at -20^0C. These differences may due to the different sample used in these two studies. Previous study used a serum sample (Halleen, 2006) while we used saliva and GCF. To date, there are no studies and information regarding the stability of enzyme activities, especially in saliva and GCF. Therefore, more studies should be conducted as sample handling can affect enzymes stability.

CONCLUSION

The findings of our study suggested that PI can stabilize enzymes activities in saliva and GCF, thus addition of PI is essential for the effective storage of AST, LDH, TRAP and ALP in samples.

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