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Stability of Human Salivary Lactate Dehydrogenase in the Present of Ethylenediaminetetraacetic Acid, Glycerol and Polyethylene Glycol at Various Temperatures: Preliminary Study

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Abstract: This study aims to determine the best storage condition for salivary lactate dehydrogenase due to decreased in native enzyme's activity after seven days of storage. Saliva samples were collected from five healthy and good oral hygiene patients. The specific enzyme activities were measured after 0 (control), 1 and 2 weeks of storage treated with 2.5 mM ethylenediaminetetraacetic acid, 10% (v/v) glycerol or 15% (w/v) polyethylene glycol at three different temperatures, i.e., room temperature, 4 and -20°C. Enzyme activity (unit mL⁻¹) was based on the rate of Nicotinamide Adenine Dinucleotide oxidations standardized at 30°C. The rate of oxidation directly proportional to enzyme's activity was measured at 340 nm. The specific activity (unit mg⁻¹) was determined through estimated protein content using Bradford analysis. The data were statistically analyzed with paired t-test based on average percentage of enzyme activities from three independent experiments. After two weeks, saliva sample in the presence of polyethylene glycol showed no significant different ($p > 0.01$) at all three temperatures compared to Lactate dehydrogenase basal activity. Lactate dehydrogenase activity of sample in the presence of ethylenediaminetetraacetic acid remained stable ($p > 0.01$) only after a week at room temperature. On the other hand, glycerol managed to stabilize salivary lactate dehydrogenase activity for two weeks at 4 and -20°C. As conclusion, polyethylene glycol showed as the best additive for salivary lactate dehydrogenase storage whereas, ethylenediaminetetraacetic acid suitable only at room temperature for a week. In addition, glycerol was suitable only in cooler conditions.

Key word: Saliva biomarker, two weeks of storage, room temperature, 4°C, -20°C

INTRODUCTION

Saliva is the most unique body fluid that can be found in the oral cavity and composed of a complex mixture of secretory product. Water is the major component of saliva that builds up 99% of its composition. In the oral cavity, saliva plays an important role in many activities, for example mastication, speech and tissue lubrication. Saliva also consists of several biological activities such as antifungal, antibacterial and antiviral (Lima *et al.*, 2010). Saliva offers an alternative biological fluid to serum that can be analyzed for diagnostic purposes. The whole saliva contains

serum-derived markers that have been found to be useful during diagnosis for variety of systemic disorders (Kaufman and Lamster, 2002). A wide range of biomarkers can be measured in saliva, such as heavy metals (e.g., lead), hormones (e.g., cortisol, dehydroxyepiandrosterone (DHEA)), enzymes (e.g., lysozyme, α -amylase), immunoglobulins (e.g., IgA) and other proteins (e.g., eosinophil cationic protein) (Koh and Koh, 2007).

Currently, saliva analysis have been used for studies in oral diseases to help assess the risk of caries (Amerongen *et al.*, 2004). Previous studies have been reported that lactate dehydrogenase (LDH) level is increased in saliva during bone remodelling due to

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orthodontic treatment (Serra *et al.*, 2003; Lee *et al.*, 2008; Perinetti *et al.*, 2002; Ariffin *et al.*, 2010). Bone remodelling is a dynamic and lifelong process in which consists of two distinct stages, i.e., resorption and formation that involved the activity of special cells known as osteoclasts and osteoblasts respectively (Abidin *et al.*, 2010). The major reason for remodelling is to enable the bones to respond and adapt to mechanical stress, such as during orthodontic treatment (Rohaya *et al.*, 2008). Abnormalities in bone remodelling occur in some of the most common diseases, such as osteoporosis and periodontitis.

LDH was known to catalyzes the reversible oxidation reaction of lactate to pyruvate and has been used as inflammation marker. LDH is a cytoplasmic enzyme that released into the extracellular environment upon cell lyses during cell death (Serra *et al.*, 2003). In dentistry, the increment of LDH activity is often related to tissue inflammation and damage commonly caused by gingivitis and periodontitis respectively (Atici *et al.*, 1998). LDH activity was found to decrease after an individual undergoes tooth cleaning treatment. LDH in saliva has been indicated suitable to be used as an indicator or diagnosis tool for periodontical defects (Numabe *et al.*, 2004).

The stability of biomarkers during storage was also another methodological issue that needs to be considered (Koh and Koh, 2007). Time during collections due to sampling and storage procedures mainly affect the enzymological activity especially involving enzymes that are temperature unstable or contamination of bacterial growth due to tedious and complex analysis procedures. Some salivary compounds or enzymes can have a very short half life that needs a shorter range of time for enzymological analysis during and after collection. However, other substance or enzymes can remained stable in saliva for a longer time, thus can be detected and quantified even after a long period of collection at standard room temperature, i.e., 25°C.

Restriction due to logistic, financial, practical and methodological reasons, during enzymological analysis has made it impossible to analyze samples immediately after collection. Therefore, a storage procedure prior to sample analysis is necessary. In this study, we investigate the stability of LDH activity from human saliva stored in three different storage mediums (ethylenediaminetetraacetic acid (EDTA), glycerol and polyethylene glycols (PEG)) at three different temperatures; room temperature, 4 and -20°C for one and two weeks.

MATERIALS AND METHODS

Samples collections: This study was conducted at 4172 and 4176 laboratories, School of Biosciences and

Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Malaysia and Orthodontic Specialist Clinic, Department of Orthodontics, Faculty of Oentistry, Universiti Kebangsaan Malaysia, malaysia from July 2008 to December 2009. In this study, saliva sample were collected from five people that fulfilled this following inclusion criteria:

- Healthy systemic condition/no systemic illness, as stated by patients
- No use of any form of anti-inflammatory drugs preceding the beginning of study and non- smokers, as stated by patients
- Good oral hygiene and periodontal health with periodontal pocket of ≤ 4 mm, full mouth plaque score (FMPS) $\leq 20\%$, full mouth bleeding score (FMBS) $\leq 20\%$ (within 15 sec after pocket depth probing)

Before the samples were collected, patients were asked to avoid doing any oral activities for 90 min. Subjects were asked to rinse up their mouth using sterile water twice prior to splitting. Saliva samples were collected into the sterile container for 10 min. Saliva samples then were centrifuged at 1000 g for 10 min in 4°C. The supernatant from centrifuged sample were used to determine the enzymes activities.

Storage of the sample: Sample was divided equally into several 1.5 mL microtubes. Each microtubes contained 500 μ L of salivary sample. The samples were stored with either 2.5 mM EDTA, 10% (v/v) glycerol, 15% (w/v) PEG or without any preservative (as a control) at room temperature, 4 or -20°C. The LDH enzyme activity of the sample was measured at 0, 1 and 2 week of storage. Each measurement of LDH activity was in triplicate.

Enzyme assay: Salivary LDH were assayed by incubation in 16.2 mmol L⁻¹ pyruvate, 0.2 mmol L⁻¹ NADH, 54.0 mmol L⁻¹ phosphate buffer (pH 7.5) and 100 μ L of saliva samples at 30°C for 5 min (Serra *et al.*, 2003) in the total volume assays of 1.0 mL. The salivary LDH activities were determined spectrophotometrically at 340 nm wavelength for every 15 sec within 3 min. In the presence of LDH, pyruvate will be reduced to L-lactate with the simultaneous oxidation of NADH into NAD⁺ and H⁺. The amount of NAD⁺ were measured spectrophotometrically and one unit of enzyme activity representing 1 μ mol of NAD⁺ released per minute at 30°C.

Protein in saliva samples were estimated according to the dye-binding method of Bradford using Bovine Serum

Albumin (BSA) as a standard (Bradford, 1976). The samples were read at wavelength 595 nm. Finally, all the enzyme activities were recorded as specific activity (unit mg⁻¹) by dividing unit of enzyme activity with protein content.

Data analysis: All the result data were statistically analysed with paired t-test by using Statistical Package for Social Sciences (SPSS) version 16.0.

RESULTS

The data showed as percentage of activity at week 1 and 2 based on week 0 that considered as 100% (Fig. 1). Week 0 representing LDH specific activity of saliva samples assayed immediately after splitting without (NATIVE) or with addition of their respective stabilizer, i.e., EDTA, PEG and glycerol. After 1 and 2 weeks, each native sample in different storage temperatures showed decreased profile of LDH activity, as compared to week 0 (Fig. 1a-c; Native). Significant different ($p < 0.01$) in percentage of specific activity at week 1 and 2 were noted as compared to control (week 0) at their respective temperature. Therefore, without any additive chemicals, salivary LDH (native) were showed to be unstable even after one week at all three temperatures (room temperature, 4 and -20°C).

The LDH specific activities analysis at room temperature showed that EDTA and PEG were suitable as additive chemicals storage at least for a week (Fig. 1a). Statistical analysis for both chemicals additive showed no significant different ($p > 0.01$) of enzyme activity at week 1 compared to week 0. However, sample in the present of EDTA for two weeks was shown to be unstable. This represented by significant decrease ($p < 0.01$) in percentage of LDH specific activity compared to control (Fig. 1a; EDTA). On the other hand, saliva samples in the presence of 15% (w/v) PEG for 2 weeks showed no significant different ($p > 0.01$) compared to week 0 (Fig. 1b; PEG). In contrast, LDH activity in 10% (v/v) glycerol (Fig. 1a; GLYCEROL) showed to be unstable either stored for 1 or 2 weeks. Statistical analysis on both storage conditions showed significant different ($p < 0.01$) of enzyme's activity at week 1 and 2 compared to their respective condition at week 0.

At 4 and -20°C, the sample in 10% (v/v) glycerol and 15% (w/v) PEG were stable in LDH activity when stored for one and two weeks (Fig. 1b and c; GLYCEROL and PEG). Statistical analysis for both samples also showed no significant different ($p > 0.01$) of enzyme's activity at week 1 and 2 compared to week 0, i.e., control. At week 2, the sample in 10% (v/v) glycerol at room temperature and

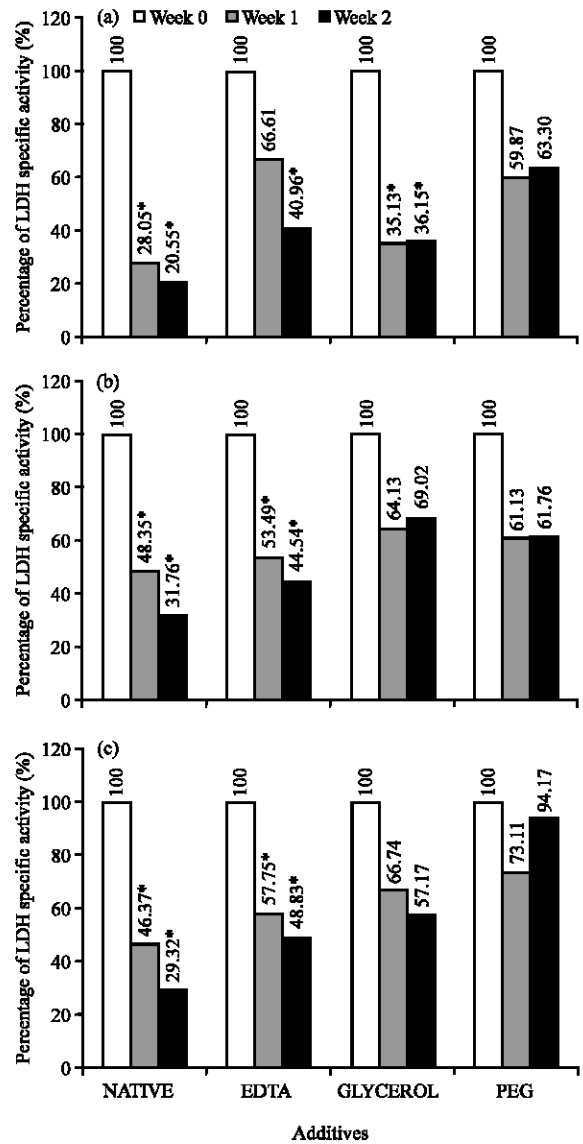


Fig. 1: Percentage of LDH activity at various temperatures and chemical additives for two weeks. Percentage of LDH specific activity after 0,1 and 2 weeks in the present of 2 mM EDTA, 10% (v/v) Glycerol, 15% (w/v) PEG and without additive (native) at room temperature (a), 4°C (b) and -20°C (c). * represent statistically significant ($p < 0.01$) using paired t-test

4°C were increased in LDH activity compared to week 1 (Fig. 1a and 1b; GLYCEROL). The same situation also occurred for samples in PEG at room temperature, 4 and -20°C (Fig. 1a, b and c; PEG). However, showed no significant different ($p > 0.01$) of LDH activity at week 1, as compared to week 2.

The LDH activity for sample in EDTA was decreased significantly to 53.49 and 44.54% for week 1 and 2 at 4°C,

respectively (Fig. 1b; EDTA). At -20°C , the average LDH activity in EDTA also decrease to 57.75 and 48.83% for week 1 and 2, respectively (Fig. 1c; EDTA). Sample in EDTA at 4 and -20°C showed significantly different ($p < 0.01$) of enzyme's activity at week 1 and 2 compared to the control, i.e., week 0. This showed that EDTA was unable to stabilize LDH activity at 4 and -20°C even for 1 week.

DISCUSSION

The experiment was performed at least in triplicate for each experiment and the results of enzyme activity were in averaged value from three independent experiments. Generally, laboratorial practice for short term storage (1-7 days) of proteins are stored at $\sim 4^{\circ}\text{C}$ in glassware or polypylene tubes that were cleaned and autoclaved and with suitable buffers. Storage at room temperature often lead to protein degradation commonly due to microbial growth. In this experiment, enzymes were mixed with either 2.5 mM EDTA, 10% (v/v) glycerol or 15% (w/v) PEG and stored at room temperature, 4 and -20°C each.

Present findings indicated that the most suitable storage condition for salivary LDH enzymatic activity was in the presence of 15% (w/v) PEG. Salivary samples stored with 15% (w/v) PEG for one and two weeks at all three temperatures (Room temperature, 4 and -20°C) showed to be not significant ($p > 0.01$) when compared to week 0 (Fig. 1a-c; PEG). Previous study by De la Pena *et al.* (2004) examined the lactate dehydrogenase activity in saliva at different temperature. They found that salivary LDH without any additional chemicals was suitable to be stored at 4°C for three months. At -20°C , they found a significant decreased in LDH activity after only 30 min of storage. When the temperature was lowered (below 0°C), ice crystal formation will occur and this could damage LDH enzyme hence lowering LDH activity (De la Pena *et al.*, 2004). De la Pena *et al.* (2004) also showed that there was no loss of activity in native samples stored at 4°C but not at -20°C . However, present study indicated that salivary LDH stored without any additional chemical (native) was significantly decreased ($p < 0.01$) after a week (Fig. 1a-c; NATIVE). Due to this contrast observation, we designed LDH stabilization experiments at different temperature but with each three different potential stabilization chemical, i.e., 2.5 mM EDTA, 10% (v/v) glycerol and 15% (w/v) PEG.

Enzymes were known to be deactivated at room temperature or even at generally suitable storage temperature, such as 4 or -20°C for cold protein storage. In addition, some enzymes will lose their activity when frozen. Freezing can lead to dramatic changes of the

protein and to a notable degree of protein inactivation. Therefore, prevention of freezing will avoid enzyme damage. However, it is possible to under cool liquids without freezing by preventing the nucleation of ice crystals. One of the approaches was by preparing the protein-containing aqueous organic emulsions. This aqueous organic emulsion able to maintain complete biological activity in the liquid state over extended periods at cool temperature such as at -20°C (O'Fagain, 2004).

One of the common organic compounds that has been used as component of solvents for enzymatic reagents stored at cool temperatures especially below 0°C is glycerol or glycerine. This was due to its character that able to suppress formation of ice crystals hence make it unfrozen or liquidify although at extreme cold temperature such as -20°C . Glycerol in enzyme solution can greatly stabilized the critical protein's biological activity although the current exact mechanism of stabilization was still not clear. In this experiment, by adding 10% (v/v) glycerol in enzyme samples will prevent solutions from freezing thus able to preserve LDH activity for two weeks at cool temperature (Fig. 1b, c; GLYCEROL).

Polyhydroxy PEG was frequently used as stabilizer to maintain protein tertiary structure. According to Yoon and Robyt (2005), PEG will bind tightly to the enzyme-proteins to give single tertiary structure that can optimally folded, hence increased the activation and stabilization of protein. In this study, PEG also showed to slightly induce the LDH activity (Fig. 1a-c; PEG). However, statistical analysis using paired t-test showed no significant different ($p > 0.01$) when compared to control, i.e., week 0 at their respective temperature. PEG molecules was showed attached to hydrophobic core of protein's tertiary structure (Rawat *et al.*, 2010). This will ensure that the non polar side chains will be packed together at the interior site of the protein to avoid these chains contacted with water, thus maintaining protein folding and therefore, the tertiary structure of the enzyme will be preserved. On the other hand, PEG also been used as agent for inducing crystallization of proteins by causing preferential hydration of proteins tertiary structures and therefore, the water will be released from the protein to facilitates protein-protein associations thus crystallization occurs (Bolen, 2004).

The presence of EDTA in saliva sample can increased the half life of the enzymes and helps to maintain the protein in a reduced state by providing copper ion and hydrogen peroxide, which can slow down the oxidation process (Andersson *et al.*, 2000). EDTA is widely used to sequester divalent and trivalent metal ions, such as Ca^{2+} and Mg^{2+} . EDTA was also known to bind metal ions, such

as Mn^{2+} , Cu^{2+} , Fe^{3+} and Co^{3+} to forms strong complexes. Complexation of these free metal ions (where previous are not essential for enzyme activity) can prevent destructive oxidation reaction of proteins' thiol groups (O'Fagain, 2004) thus can avoid proteolysis and stabilized enzyme's activity, such as salivary LDH. However, in present study, EDTA was showed to be able to stabilize the LDH activity only for a week at room temperature (Fig. 1a: EDTA). An early indication that in saliva, formation of free metal ion complexes or sequestering of divalent and trivalent metal ion were not occur in cool temperature such as 4 and $-20^{\circ}C$.

In contrast with De la Pena *et al.* (2004) that showed native salivary LDH was stable for 3 months at room temperature, 4 and $-20^{\circ}C$, our observation showed that LDH was unstable after seven days at their respective temperatures. In addition, we also conducted LDH stability study that involved three common additive chemicals for enzymes stability, i.e., 2.5 mM EDTA, 10% (v/v) Glycerol and 15% (w/v) PEG.

CONCLUSION

Stabilizer is required to store salivary LDH and PEG is the best additive to store this enzyme for two weeks at room temperature, 4 and $-20^{\circ}C$. In addition, salivary LDH treated with EDTA stable at room temperature for a week, while salivary LDH in glycerol was stable at 4 and $-20^{\circ}C$ for 2 weeks.

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