



Total esterase measurement in saliva of pigs: Validation of an automated assay, characterization and changes in stress and disease conditions

Fernando Tecles^a, María D. Contreras-Aguilar^a, Silvia Martínez-Miró^b, Asta Tvarijonaviciute^a,
Silvia Martínez-Subiela^a, Damián Escribano^a, José J. Cerón^{a,*}

^a Interlab-UMU, Campus of Excellence Mare Nostrum, University of Murcia, 30100, Spain

^b Animal Production Department, Campus of Excellence Mare Nostrum, University of Murcia, 30100, Spain

ARTICLE INFO

Keywords:

Disease
Esterase
Pig
Saliva
Stress

ABSTRACT

An automated spectrophotometric method for total esterase activity (TEA) measurement in porcine saliva has been developed and validated, using 4-nitrophenyl acetate (4-NA) as substrate. The method was precise and accurate, with low limit of detection, and was able to measure samples with TEA activities up to 400 IU/L without any dilution. In addition, the different enzymes contributing to TEA were characterized, being identified carbonic anhydrase VI (CA-VI), lipase, cholinesterase (ChE) and cholesterol esterase (CEL). TEA significantly increased (1.49-fold, $P < 0.01$) in healthy pigs just after applying an acute stress stimulus consisting of nasal restraint, being lipase and cholinesterase the main responsible of this increase. TEA was significantly increased (1.83-fold, $P < 0.001$) in a group of pigs with lameness; in this case, in addition to lipase and ChE, CA-VI also increased. The results found in this report indicate that TEA can be easily measured in porcine saliva with an accurate and highly reproducible automated method. Salivary TEA is mainly due to the activity of four enzymes: CA-VI, lipase, ChE and CEL, and these enzymes can change in a different way in situations of stress or disease.

1. Introduction

The use of saliva as a sample has several advantages compared to blood, since it can be collected easily and non-invasively by individuals with limited training (Bulgaroni et al., 2014). These advantages are of particular importance in pigs, in which blood sampling is technically difficult to perform and stressful for the animal. Several salivary biomarkers related with animal welfare such as cortisol (Escribano et al., 2012), chromogranin A (CgA) (Escribano et al., 2013; Ott et al., 2014) or alpha-amylase (sAA) (Fuentes et al., 2011) have been successfully measured in pigs. Therefore, saliva could be considered as a suitable sample for the measurement of welfare biomarkers in this species.

Saliva has an abundant esterase activity that can derive from various sources such as carbonic anhydrase isozyme VI (CA-VI) (Supuran, 2008; Koç Öztürk et al., 2012), cholesterol esterase (Finer et al., 2004), cholinesterase (ChE), carboxylesterase, lipase or even albumin (Ceron et al., 2014). Recently, a method for total esterase activity (TEA) measurement has been reported in human saliva using 4-nitrophenyl acetate (4-NA) as substrate. In addition, the different analytes that contribute to TEA in human saliva have been characterized, being CA-VI the main responsible of TEA, and it was also observed

that physical stress increased TEA in saliva (Tecles et al., 2016b).

To the authors' knowledge, TEA has not been studied in porcine saliva. The first aim of this study was to validate an automated method for TEA determination in porcine saliva. In addition, this study had the purposes of characterize TEA by the identification of the different compounds that are involved in this activity, and evaluate the possible changes of this enzyme in a experimental model of restraint stress and in a specific disease (lameness) that can be frequently found in pig farms.

2. Material and methods

2.1. Animals

Saliva was collected from crossbred growing pigs (*Sus scrofa domestica*) Large White × Large White. All animals were vaccinated against *Mycoplasma hyopneumoniae* (Stellaune Mycoplasma, inactivated *Mycoplasma hyopneumoniae* NL 1042, Pfizer Animal Health) and Porcine circovirus type 2 (Porcilis® PCV, MSD Animal Health, Boxmeer, The Netherlands) during the phase of lactation. They were in the last phase of fattening with 2–3 months of age and were housed in the Experimental Farm of the University of Murcia (Murcia, Spain).

* Corresponding author.

E-mail address: jjceron@um.es (J.J. Cerón).

Pigs were given ad libitum access to a nutritionally balanced diet and water. The animals were housed in pens with a minimum space of 0.65 m² per animal (Council Directive 2001/88/CE of 23 October 2001 amending Directive 91/630/CEE concerning minimum standards for the protection of pigs). The temperature in the pens was kept at a maximum of 23 °C and a minimum of 18 °C.

2.2. Sampling

Saliva was collected using Salivette tubes (Sarstedt, Aktiengesellschaft & Co. D-51588 Nümbrecht, Germany) containing a sponge instead of cotton swab (because these were less absorbent and released more saliva following centrifugation). The sampled pigs were allowed to chew the sponge, which was clipped to a flexible thin metal rod, until thoroughly moist. The sponges were then placed in test tubes and centrifuged at 2170g at 4 °C for 10 min. Finally, saliva samples (approximately 0.5–1.0 mL per sponge) were stored in Eppendorf tubes and frozen at –80 °C until analysis.

2.3. Spectrophotometric assay for TEA determination

TEA was analyzed as previously reported (Tecles et al., 2016b). Briefly, the method was based on the hydrolysis of the substrate 4-NA (Sigma-Aldrich Co, St Louis, Mo, USA) by salivary esterases. The rate of generation of the product of the 4-NA hydrolysis, 4-nitrophenol, was determined at 405 nm. The nonenzymatic hydrolysis of 4-NA was subtracted from the total hydrolysis rate. The activity, expressed in IU/L, was based on the molar absorptivity (14,000 M^{–1} cm^{–1}) of 4-nitrophenol at 405 nm, pH 7.4. The method was adapted to an automated biochemical analyzer (Olympus AU400, Olympus Diagnostica GmbH, Ennis, Ireland).

2.4. Analytical validation of TEA assay

Two pools of saliva, one integrated by 5 saliva samples with a low esterase value and the second one integrated by 5 saliva samples with a high esterase value, were used for the analytical validation of the assay, which included:

- Reproducibility. Within- and between-run precision was calculated. To establish within-run precision, replicates of two pools of saliva samples were analyzed six times in one day. Between-run precision was determined analysing the same pools on six different days within two weeks. Coefficients of variation (CV) were calculated as mean of the replicates divided by standard deviation plus 100.
- Linearity under dilution. Samples from each of the 2 pools of saliva were serially diluted with sample buffer and assayed. The results were compared with those expected by linear regression analysis.
- Limit of detection. It is defined as the lowest concentration of an analyte that could be distinguished from a specimen with a value of zero, and it was calculated on the basis of data from 20 replicate determinations of the zero standard (assay buffer), as the mean value plus 2 standard deviations.

2.5. Characterization of the contribution of different esterases on TEA measured with 4-NA

In order to identify the compounds that can contribute to TEA activity, the following experiments were performed:

- Effect of the inhibitor diisopropylfluorophosphate (DFP, Sigma-Aldrich Co) on TEA. DFP is an organophosphate compound capable of inhibit enzymes such as cholinesterase, carboxylesterase and cholesterol esterase (Momsen and Brockman, 1976; Dettbarn et al., 1999). A total of six saliva samples obtained from healthy subjects were separated in 4 aliquots. Three aliquots from each different

sample were inhibited by adding DFP at different concentrations. An equal amount of diluent was added to the fourth aliquot. Final DFP concentrations in the four aliquots of each sample were 0, 5×10^{-4} , 1×10^{-3} and 1×10^{-2} M. All aliquots were incubated at room temperature for 30 min. Then, esterase activity was measured as described above.

- Effect of the inhibitor Orlistat (tetrahydrolipstatin, Sigma-Aldrich Co). Samples were incubated with the lipase inhibitor Orlistat to assess contribution of lipase to TEA. To prepare Orlistat for the assay, the compound was diluted in dimethylsulfoxide (DMSO, Sigma-Aldrich Co). Six saliva samples obtained from healthy subjects were separated in 3 aliquots. Two aliquots were inhibited by adding Orlistat at different concentrations. The third aliquot was incubated with an equal amount of the diluent. The final Orlistat concentrations in samples were 0, 2.5×10^{-4} and 5.0×10^{-4} M. All aliquots were incubated at room temperature for 30 min. Then, TEA was measured as described above.
- Effect of the combination of the inhibitors DFP and Orlistat. Six saliva samples obtained from healthy subjects were separated in 2 aliquots. One aliquot was inhibited by adding DFP and Orlistat at final concentrations of 1×10^{-2} M and 2.5×10^{-4} M, respectively. The second aliquot was incubated with an equal amount of the diluent. All aliquots were incubated at room temperature for 30 min. Then, TEA was measured as described above, and values obtained with the original method and that combining both inhibitors (TEA_{DFP + Or}), which theoretically measures CA-VI, were compared.
- Effect of the addition of lipase. Commercially available human lipase (232 IU/L, Beckman Coulter Lipase calibrator, Beckman Coulter Inc., CA, USA) was serially diluted with distilled water. Six saliva samples obtained from healthy animals were separated in 7 aliquots. Lipase dilutions were added to six aliquots from each different sample, giving the following added activities: 2.9, 5.8, 11.6, 23.2, 58.0 and 116.0 IU/L. The same volume of distilled water was also added to the seventh aliquot. TEA was measured before and after lipase addition. Additionally, lipase activity was analyzed in the 6 saliva samples in order to assess its possible correlation with TEA.
- Effect of the addition of albumin. Bovine serum albumin (BSA, Sigma-Aldrich Co) was added to aliquots from 6 fresh saliva samples at increasing concentrations (0.05, 0.5, 0.75, 1.0, 2.5 and 5.0 mg/mL). The same volume of distilled water was also added to a seventh aliquot. TEA was measured before and after albumin addition.
- Western blot for CA-VI and CEL. A rabbit polyclonal antibody against CA-VI (anti-Carbonic Anhydrase VI (C-term) antibody, antibodies-online GmbH, Aachen, Germany) and a mouse polyclonal antibody against CEL (CEL E-4, Santa Cruz Biotechnology, Inc.) were used. Although the antibodies were developed against the human protein, according to the manufacturers both antibodies have cross-reactivity with the proteins in pigs. The analysis protocol was as follows: 50 µg of protein from a pool of porcine saliva was resolved in 12% homemade polyacrylamide gel (Bio-Rad Laboratories Inc., Hercules, CA, USA), and transferred to nitrocellulose membranes (Bio-Rad Laboratories Inc.) by electroblotting. Membranes were incubated with the antibody as stated in the text, following HRP-conjugated secondary Ab (goat HRP- conjugated secondary anti-rabbit IgG, Abcam, Cambridge, UK; and goat HRP- conjugated secondary anti-mouse IgG, ThermoFisher Scientific Inc., Waltham, USA; respectively for anti-CA-VI and anti-CEL) at 1:2000 dilution incubation and detection using Pierce ECL2 kit (Pierce, Thermo Fisher Scientific, USA) and Thyphoon 9410 scanner (GE Healthcare, Wilmington, MA, USA).
- Enzyme linked immunoassay (ELISA) for CA-VI. An ELISA was developed using a goat polyclonal IgG anti-human CA-VI (CA VI N-17, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) as primary antibody, and a HRP- conjugated anti-goat IgG as secondary anti-

Download English Version:

<https://daneshyari.com/en/article/5543944>

Download Persian Version:

<https://daneshyari.com/article/5543944>

[Daneshyari.com](https://daneshyari.com)