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An enzyme sensor for the determination of total amines in dry-fermented sausages

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ABSTRACT

An enzyme sensor employing diamine oxidase from porcine kidney in combination with an oxygen electrode has been developed and optimised to estimate the content of total amines in dry-fermented sausages. The enzyme DAO was immobilised on a preactivated Immunodyne membrane using glutaraldehyde as cross-linking agent. The enzymatic determination was based on the measurement of the consumed O_2 in a platinum electrode poised at −600 mV versus Ag/AgCl. The reaction was started by the direct injection of either the standard or meat extract on the enzymatic membrane. The immobilised enzyme could be used up to 30 analyses along the day without significant loss of sensitivity and could be stored at 4 °C showing good stability for at least 6 weeks. A good correlation was observed when comparing data obtained with the enzyme sensor to those obtained with a standard HPLC method. Thus, this sensor may be a reliable screening method to detect the presence of biogenic amines in dry-fermented sausages that can constitute a useful tool for quality control in the meat industry.

1. Introduction

Biogenic amines are basic nitrogen compounds usually formed by the decarboxylation of their precursor amino acids (Silla-Santos, 1996). The formation of biogenic amines in the food has been applied as a parameter for the evaluation of food quality because the intake of foods containing high concentrations can constitute a health hazard (Ruiz-Capillas and Jiménez-Colmenero, 2004). The presence of biogenic amines as microbial metabolites is associated to food fermentation processes (Vidal-Carou et al., 2007). In the case of cured products, high quantities of certain biogenic amines can be observed in the final products as a consequence of the use of poor quality raw material, microbial contamination and inadequate conditions during processing and storage (Bover-Cid et al., 2006). The levels of biogenic amines in fermented sausages may present large variations depending on the type of products, manufacturers and even from batch to batch (Vidal-Carou et al., 2007). Several studies have been focused during the last decades on different techniques to analyse biogenic amines in dry-fermented sausages (Lu et al., 2010; Latorre-Moratalla et al., 2009; Önal, 2007). Among them, methods based on the use of high-performance liquid chromatography (HPLC) have been the most used. However, the amperometric enzyme biosensor has recently appeared as a suitable alternative due to its simplicity, quickness and low cost (Mello and Kubota, 2002; Alonso-Lomillo et al., 2010). Furthermore, a similar enzyme sensor using immobilised xanthine oxidase has been recently developed for the measurement of pork meat freshness (Hernández-Cázares et al., 2010) and evolution of dry-cured ham (Hernández-Cázares et al., 2011). Thus, the aim of this study was to develop a method based on an enzymatic sensor with immobilised diamine oxidase to determine the content of total amines in dry-fermented sausages.

2. Materials and methods

2.1. Chemicals and reagents

Diamine oxidase (DAO) (EC 1.4.3.6 from pig kidney, 0.18 U/mg of solid), glutaraldehyde (50%), glycine, histamine, cadaverine, putrescine, tyramine, agmatine, tryptamine, spermine, spermidine were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol, perchloric acid, potassium carbonate, 2-mercaptoethanol were purchased from Scharlau (Barcelona, Spain). Sodium acetate, boric acid, glacial acetic, potassium hydroxide, Brij-35 (30%) were purchased from Panreac (Panreac Química, S.A., Barcelona, Spain). The Immunodyne ABC membrane (Nylon 6.6, pore size 0.45 μm) was supplied by Pall Europe (Portsmouth, United Kingdom), the Teflon membrane (12.7 μm) by Thermo Fisher Scientific Inc. (Madrid, Spain) and the Ultrafree-CL high-flow Biomax-PB polyethersulphone membrane (100 kDa) filters by Millipore Corporation (Bedford, MA, USA).

2.2. Dry-fermented sausages preparation and sampling

Dry-fermented sausages were prepared with lean pork (80%) and pork fat (20%) and 27 g of sodium chloride, 20 g of lactose, 20 g of dextrin, 20 g of sodium caseinate, 7 g of glucose, 0.5 g of

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sodium ascorbate and 0.15 g of sodium nitrite per kilogram to meat mixture. The mixture was ground to 10-mm particle size, vacuum homogenised in two alternating cycles of 2 min each and inoculated with a commercial starter culture SP-318 containing *Lactobacillus sakei, Pediococcus pentosaceus, Staphylococcus xylosus* and *Staphylococcus carnosus* (from Danisco, Cultor, Madrid, Spain). Finally the meat mixture was stuffed into 75–80 mm diameter collagen casings (Fibran, S.A., Girona, Spain), with an approximate final weight of 500 g. The sausages were kept for 24 h in a chamber at 3–5 °C in order to homogenise all ingredients and additives. Then, they were dried for 10 days at 9 °C, 10 days at 11 °C and 21 days at 9 °C with 85–75% relative humidity and vacuum packed and stored at 4 °C up to 92 days.

The curing process was followed by performing microbial, weight loss, moisture and pH analysis (Marco et al., 2006).

2.3. Amines extraction and deproteinisation

Dry-fermented sausages samples were submitted to a simultaneous extraction and deproteinisation following the method described by Burns and Ke (1985) with some minor changes. Five grams of dry-fermented sausages were ground and homogenised with 15 mL of cold 0.6 M perchloric acid for 10 min at 4 °C in a stomacher (IUL Instrument, Barcelona, Spain). The extract was centrifuged at 10,000*g* for 20 min at 4 °C and the supernatant was filtered through glass wool. Twelve millilitres of this solution was neutralised (up to pH 6.5–7.0) by adding solid potassium carbonate and letting it to stand in an ice bath for 20 min. The neutralised extract was centrifuged again as above, and the supernatant was stored at −20 °C until use. The extracts were thawed and centrifuged again for 5 min before further analysis.

2.4. HPLC analysis of amines

Amines were determined by ion-pair high-performance liquid chromatography (IP-HPLC) and post-column derivatisation with ortho-phthalaldehyde (OPT), according to the method described by Hernández-Jover et al. (1996). The chromatographic separation was carried out using a Nova-Pack C_{18} column, 3.9 × 150 mm and 4 μm particle size (Waters Chromatography, Milford, MA). The separated compounds were monitored using a fluorescence detector set at 340 nm excitation and 445 nm emission wavelengths and identified by their respective retention time. The quantification was performed by external standard method using respective calibration curves.

2.5. Enzyme sensor analysis for the total amine content

The amperometric measurement of total amines was carried out using an enzyme sensor based on a membrane with immobilised diamine oxidase (DAO) coupled inside an oxygen electrode assembly model 20 Dual Digital (Rank Brothers, Bottisham, Cambridge, England). The potential of polarisation was fixed at −600 mV with respect to the Ag/AgCl reference electrode and the current output was recorded by a data-logger ADC-16 (Tip Technology Limited, St. Net. Cambridgeshire, United Kingdom).

2.5.1. DAO immobilisation

The DAO immobilisation method consisted in depositing drop by drop on a preactivated Immunodyne ABC membrane (1 cm^2), 5 μL of an enzyme solution (110 mg DAO/mL of 100 mM sodium phosphate buffer, pH 7.2), previously concentrated by using a 100,000 Da Ultrafree-CL centrifugal filter device (4470 rpm for 40 min). The membrane with the enzyme was air-dried for 15 min, immersed in a glutaraldehyde solution (1%) for 30 s and immediately washed with a glycine solution (100 mM in sodium phosphate buffer, pH 7.2) to eliminate any unreacted glutaraldehyde. The DAO immobilised membrane was applied to an oxygen permeable Teflon membrane placed in the oxygen electrode and clamped into the thermostated reaction cell that was kept at 30 °C. Forty microlitres of an appropriate dilution of amine standard or sausage extract was injected directly on the DAO-membrane. After each measurement, the enzyme sensor was washed with the sodium phosphate buffer and dried with paper tissue. As a result of the catalytic action of DAO on amines, which conveys oxygen consumption, the decrease of current (mA) at 50 s was used to detect the total amine in the enzyme sensor.

2.5.2. Enzyme sensor evaluation

DAO catalyses the oxidative deamination of amines and diamines to the corresponding aldehydes and ammonia accompanied by a reduction of molecular oxygen to hydrogen peroxide, as follows:

$$RCH_2NH_2 + O_2 + H_2O \xrightarrow{DAO} RCHO + H_2O_2 + NH_3$$

Once either the standard or the meat extract is injected into the enzyme sensor, the reaction takes place and the current decreases by the oxygen consumption, in a proportional way to the amine content.

The operational stability was tested by successive injections of histamine standard (0.01 mg/mL) along 2 days. For the storage stability, 10 DAO membranes were stored in sodium phosphate buffer (100 mM, pH 7.2) at 4 °C. One DAO membrane per week was tested with the histamine standard (0.01 mg/mL) per triplicate.

The repeatability of the analysis was tested by repeated injections of the same histamine standard and of a fermented sausage extract (42 days of curing), which were all analysed on the same day with the same equipment under the same operating conditions.

3. Results and discussion

3.1. Enzyme immobilisation and optimisation

The immobilisation method is one of the most important parameters in the development of the enzyme sensor, because it affects the enzyme capability to act. The commercial DAO exhibited low enzymatic activity (0.18 U/mg of solid) and thus required a concentrating step using cut-off membrane filters. The use of glutaraldehyde allows high enzyme loading on the membrane and is reported to improve the operation of the sensor (Carelli et al., 2007) even though Karube et al. (1980) mentioned that the DAO can be easily denatured in the presence of glutaraldehyde. Thus, some caution and optimisation of the immobilisation was required. Several enzyme and glutaraldehyde concentrations, ranging from 5 μL of 60–120 mg DAO/mL and 1–2.5%, respectively, were used (Male et al., 1996; Bouvrette et al., 1997; Carelli et al., 2007). When using high concentrations of both, enzyme and glutaraldehyde, the polymerisation occurred very fast and it was impossible to pipette the mixture on the membrane. On the contrary at low concentrations of both, homogeneous mixtures were formed which could be easily deposited on the membrane but, after drying, there was an incorrect diffusion of oxygen through the thin film and very low current signals were obtained giving poor reproducibility.

It was also observed a rapid leak of the DAO activity, possibly because of its low initial enzyme activity and/or denaturation when adding the glutaraldehyde (Karube et al., 1980). In order to obtain an increased activity of the enzyme, two strategies were followed. First, the enzyme solution was concentrated as mentioned above. Second, the negative effect of the glutaraldehyde was minimised by a short immersion (30 s) of the membrane with the enzyme, in a 1% glutaraldehyde solution on the opposite side of the