Chapter 21

Biosensors for D-Amino Acid Detection

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Abstract

The presence of D-amino acids in foods is promoted by harsh technological processes (e.g., high temperature or extreme pH values) or can be the consequence of adulteration or microbial contamination (D-amino acids are major components of the bacterial cell wall). For this reason, quality control is becoming more and more important both for the industry (as a cost factor) and for consumer protection. For routine food analysis and quality control, simple and easily applicable analytical methods are needed: biosensors can often satisfy these requirements. The use of an enzymatic, stereospecific reaction could confer selectivity to a biosensor for detecting and quantifying D-amino acids in foodstuffs. The flavoenzyme D-amino acid oxidase from the yeast *Rhodotorula gracilis* is an ideal biocatalyst for this kind of application because of its absolute stereospecificity, very high turnover number with various substrates, tight binding with the FAD cofactor, and broad substrate specificity.

Furthermore, alterations in the local brain concentrations of D-serine (predominantly D-amino acid in the mammalian central nervous system) have been related to several neurological and psychiatric diseases. Therefore, quantifying this neuromodulator represents an important task in biological, medical, and pharmaceutical research. Recently, an enzymatic microbiosensor, also using *R. gracilis* D-amino acid oxidase as biocatalyst, was developed for detecting D-serine *in vivo*.

Key words: Amperometric detection, D-Amino acid oxidase, D-Serine, Food quality, Analytical detection

1. Introduction

Microorganism-synthesized D-amino acids are estimated to constitute approximately one third of the D-amino acid burden in humans (1). Moreover, naturally occurring L-amino acids may be transformed to their mirror image configuration isomers through exposure of food proteins to processing conditions such as high pH, heat, and acids. The presence of D-amino acids in food is normally associated with a decrease in protein digestibility, thus affecting the bioavailability of essential amino acids and ultimately impairing the

Loredano Pollegioni and Stefano Servi (eds.), *Unnatural Amino Acids: Methods and Protocols*,
Methods in Molecular Biology, vol. 794, DOI 10.1007/978-1-61779-331-8_21, © Springer Science+Business Media, LLC 2012

nutritional value of food (2). Both the concentration of D-amino acids and the D/(D+L) ratio have been proposed for assessing food quality and identifying food adulteration (2, 3). Indeed, D-amino acid concentrations in food often depend on the original content of these "atypical compounds" in the raw material: D-Ala, D-Glu, D-Asp, D-Lys, D-Ser, D-Pro, D-Phe, D-Arg, and D-Leu have been identified as natural components of various foods (2). Significant levels of these D-amino acids were measured in bread (4), coffee (5), fruit and vegetable juices (6), honey, chocolate (7), vinegars (8), wine (9), cheese (10), and dairy products (11, 12). In particular, raw milk from ruminants contains D-Ala, D-Asp, D-Glu, D-Lys, and D-Ser. Relatively high concentrations of these D-amino acids are also present in widely consumed fermented milk products, among which ripened cheeses are the richest in D-amino acids (10–12). The D-amino acid content differs in different cheeses and also changes according to means of production and storage. For example, Parmigiano Reggiano and Grana Padano cheeses can be distinguished from each other by the relative amounts of D-Ala, D-Asp, and D-Glu, while the racemization degree, the D/(D+L) isomer ratio, can be used to estimate the sample age (11). Furthermore, all D-amino acids can be found in foods as a consequence of adulteration, e.g., when hydrolyzed proteins are added to mask low nutrient content (11). For these reasons, determination of the overall D-amino acid content in food specimens constitutes an adequate analytical parameter.

Generally, efficient enantiomeric separation and analysis procedures using high performance liquid chromatography, gas chromatography, or capillary electrophoresis are performed to detect the presence of the different amino acids in foodstuffs, beverages, and biological samples (2, 12). Although these analytical methods are very sensitive, reliable, and reproducible, they are time-consuming and expensive (requiring specialized personnel) and often too slow for the food industry. For routine and quality control measurements, simple and easily applicable analytical methods are needed: biosensors can frequently satisfy these requirements.

During the last decade, many research groups have developed enzyme sensors that specifically detect D-amino acids based on the stereospecific, oxidative deamination catalyzed by the FAD-containing flavoenzyme D-amino acid oxidase (EC 1.4.3.3, DAAO): DAAO converts D-amino acids into the corresponding α-keto acids and ammonia using molecular dioxygen and producing hydrogen peroxide; for a review, see refs. 13, 14. Currently, the most efficient biosensors use the enzyme from the yeast *Rhodotorula gracilis* (RgDAAO), which exhibits a very high turnover number, a broad substrate specificity, and a tight binding with the FAD cofactor (13, 14). Moreover, the possibility of employing engineered RgDAAO variants with an enlarged substrate specificity and, in particular, with a remarkably improved catalytic efficiency on acidic and basic substrates (15–17) renders "the latest-generation

biosensors" powerful analytical tools for determining the total D-amino acid content, even on complex matrices (17, 18).

2. Materials

2.1. Enzymes

1. Recombinant wild-type (19) and M213R, M213G, and T60A/Q144R/K152E variants of RgDAAO were overexpressed in *Escherichia coli* BL21(DE3)pLysS cells using the pT7-HisDAAO expression vector and purified by Hitrap Chelating chromatography because of the addition of an His-tag at the N-terminus, as detailed in refs. 15–17. The enzyme concentration is determined using an extinction coefficient of ~12.6/mM/cm at 455 nm. The kinetic parameters of different purified DAAO variants were determined at pH 8.5 and 25°C. The specific activity on D-Ala as a substrate is: 98 U/mg protein (wild-type RgDAAO), 6.6 U/mg protein (M213R), 19.2 U/mg protein (M213G), and 92 U/mg protein (T60A/Q144R/K152E). One DAAO unit corresponds to the amount of enzyme that converts 1 μmol of D-amino acid per minute at 25°C.
2. The DAAO variants used with the Specialities biosensor were covalently immobilized on an Amberzyme Oxirane support (Rohm and Haas Advanced Biosciences, USA) by a coupling procedure involving protein-free amino groups (17). The activity of the immobilized enzyme was determined by an amperometric assay (oxygen electrode), using a weighted amount of matrix (100 mg/mL) in 100 mM potassium phosphate, pH 8.0.
3. The final RgDAAO preparation used for the microbiosensor was concentrated up to 58 mg/mL in a solution containing 25 mg/mL bovine serum albumin and 1% glycerol in 20 mM phosphate buffer, pH 8.5. The enzyme was immobilized under saturated glutaraldehyde vapors (20).

2.2. Midaspro Amperometric Biosensor (15, 18)

1. Midaspro biosensor (Sartorious A.G., Germany).
2. Calibration solution: 0.2 mg/mL hydroquinone.
3. Working buffer solution: 100 mM disodium pyrophosphate, pH 8.5.
4. Substrate standard solutions: 0.15–3 mM D-Ala or 0.5–20 mM D-Asp in working buffer.
5. Washing solution: 1% sodium hypochlorite.

2.3. Specialities Amperometric Biosensor (17)

1. Electrochemical cells (Specialities s.r.l., Italy).
2. Data acquisition and processing system.
3. Working buffer solution: 100 mM potassium phosphate, pH 7.0.

4. D-Ala standard solutions: 0.25–5 mM in 100 mM potassium phosphate, pH 7.0.

2.4. Microbiosensor (20)

1. The biosensor is constituted of a 90% Pt/10% Ir wire of 25 µm in diameter (Goodfellow, UK) glued to a 0.3-mm copper wire using electroconductive silver paint (Radiospares, Beauvais, France) and inserted into a pulled glass capillary (Harvard Apparatus, UK).
2. Patch-clamp amplifier (Geneclamp GC500, Molecular Devices, USA) or electrochemistry amplifier (VA-10, NPI Electronics, Germany) used with a two-electrode potentiostat.
3. Acquisition board (ITC-18, Instrutech, USA).
4. Reference electrode: chlorided silver wire electrode placed directly in the recording chamber.
5. D-Ser Standard solutions: 0.5–10 µM in 10 mM PBS (phosphate-buffered saline constituted by 8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, and 0.24 g/L KH_2PO_4), pH 7.4.
6. Artificial extracellular medium: 126 mM NaCl, 1.5 mM KCl, 1.25 mM KH_2PO_4, 1.5 mM $MgSO_4$, 2 mM $CaCl_2$, and 10 mM HEPES, pH 7.4.

3. Methods

3.1. Determination of Neutral and Acidic D-Amino Acid Content (Midaspro Biosensor)

The electrochemical measurements are carried out at room temperature in a 5-mL amperometric cell consisting of a plastic flow cell that contains a working and a reference electrode at an applied fixed voltage of +400 mV vs. Ag/AgCl (15, 18). The enzyme (approximately 8–25 µg of protein) is immobilized by simple absorption on the surface of the working electrode (a graphite disk 20 mm in diameter and 2 mm thick, Fig. 1).

The assays employ two RgDAAO variants displaying a different substrate specificity: (a) the wild-type enzyme, which is active on neutral and polar D-amino acids but inactive on the acidic ones (the catalytic efficiency, expressed as the k_{cat}/K_m ratio, is ~4,000-fold higher on D-Ala than on D-Asp as a substrate) (13, 14) and (b) the M213R variant, which is active on D-Ala as well as on D-Asp and D-Glu (15).

The detection procedure is performed as follows:

1. Calibrate the electrochemical cell that was previously filled with 5 mL of the working buffer solution: when the anodic signal is stable, inject 20 µL of the calibration solution and measure the corresponding current intensity (calibration peak).
2. Wash the calibrated electrode with 5 mL of the washing solution and 20 mL distilled water.

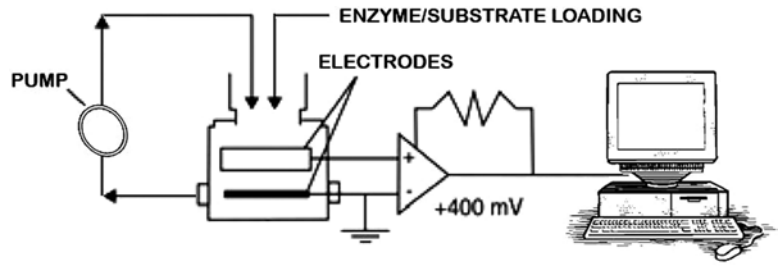

Fig. 1. Scheme of the amperometric biosensor Midaspro (15, 18). By using the peristaltic pump, the substrate/sample solutions can be efficiently mixed through the electrochemical cell. The signal is recorded at a fixed potential of +400 mV.

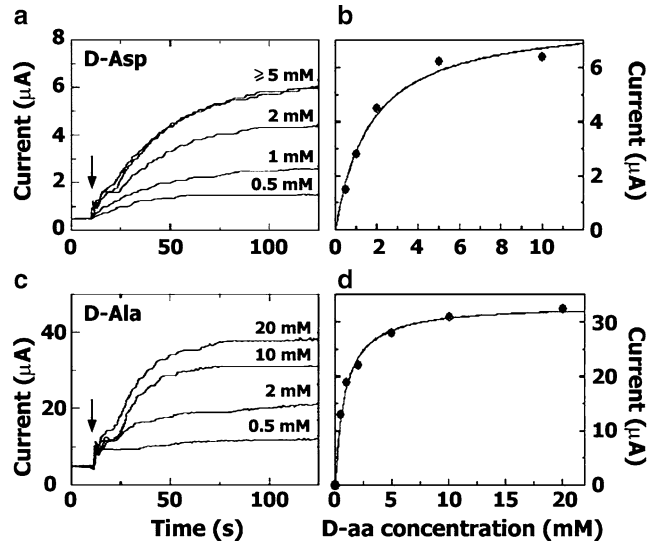

Fig. 2. Amperometric traces obtained with the Midaspro biosensor and M213R RgDAAO as biocatalyst and using standard solutions at different concentrations of D-Ala (**a**) and D-Asp (**c**). The corresponding calibration curves are depicted in the right panels (**b**, **d**).

3. Use standard solutions of substrate (D-Ala and D-Asp for wild-type and M213R RgDAAO, respectively) to generate a calibration curve: 5 mL of 0.5–20 mM D-Ala or D-Asp solutions are added in the electrochemical chamber.
4. When the recorded current is stable, inject 1.5 U of wild-type or 0.2 U of the M213R variant RgDAAOs.
5. Plot the current values detected after 3 min of recording with the different standard solutions (Fig. 2a, c) as a function of substrate concentration and fit the data points by a classic Michaelis–Menten equation (Fig. 2b, d). See Note 1.
6. At the end of the recording time, rinse the cell with 5 mL of washing solution (see Note 2).
7. For the analytical measurement, fill the electrochemical cell with 4 mL of working buffer solution and 1 mL of a biological

sample containing an unknown concentration of D-amino acid. When the recorded current is stable, inject 1.5 U of wild-type or 0.2 U of M213R variant RgDAAOs.

8. The amount of neutral or acidic D-amino acids is measured from the current intensity by using the calibration curves (Fig. 2b, d). See Notes 3 and 4.

3.2. Determination of the Total D-Amino Acid Content (Specialities Amperometric Biosensor)

The assay is performed by using several electrochemical cells, produced by a screen-printing process and which contain both a working electrode (a graphite disk, 28 mm in diameter) and a reference electrode (Ag/AgCl) (17). This device represents an improved version of the previous biosensor and relies on very close contact between the immobilized biocatalyst and the electrode surface. The response is calculated by the difference between signals from the working and the reference electrodes, the latter indicating the presence of the enzyme (see Notes 5 and 6). The engineered M213G and T60A/Q144R/K152E RgDAAO variants represent the best catalysts available (better if used simultaneously) to determine the total D-amino acid content in biological samples because they respond to all (neutral, acidic, and basic) D-amino acids (17). The T60A/Q144R/K152E RgDAAO variant exhibits the best correlation between theoretical activity values on various D-amino acid mixtures and experimental results (see Notes 7 and 8). Moreover, variability in response as a function of the D-amino acid composition is low for RgDAAO variants in the immobilized form (17), see Note 6. Due to the additive nature of the individual amino acid responses, this device can be used to analyze solutions containing both single species and complex D-amino acid mixtures.

The assay is performed as follows.

1. Fill both the electrochemical chambers with 3 mL of a 1-mM D-amino acid solution in working buffer solution.
2. Wait for a few minutes until the current trace is stable and add 0.5–4.0 U of M213G and/or T60A/Q144R/K152E RgDAAO variants in the working cell, but not in the reference one.
3. Record the amperometric response until it reaches a plateau value (~10 min): the intensity of the recorded current is directly related to the D-amino acid concentration.
4. Following each measurement, the screen-printed electrodes are discarded and replaced with new ones.
5. Use standard solutions of D-Ala (in the 0.25–5 mM concentration range) to generate a calibration curve.

Please note that this biosensor has been used to detect D-amino acids in complex samples such as cheese specimens (17). In this case, the procedure is as follows:

1. Suspend finely grated Grana Padano cheese in working buffer solution at a 0.1 mg/mL final concentration.

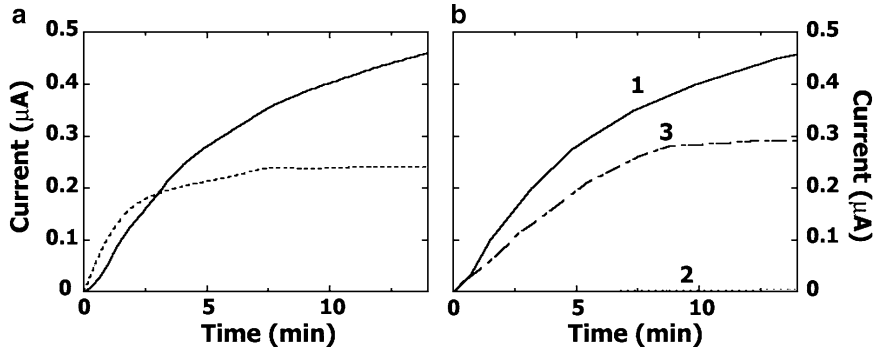

Fig. 3. Determination of D-amino acid content in cheese samples using the Specialities Amperometric Biosensor (18). (**a**) Response of the biosensor while measuring total D-amino acids content in Grana Padano cheese samples (*line*, 2 mL; *dotted line*, 1 mL). (**b**) The 2 mL cheese sample (*line*, 1) was treated for 20 min with 1.5 U of RgDAAO before measurement (*dotted line*, 2). Then, 1 mM D-Ala (*dashed line*, 3) was added to this sample, and after 10 min, it gave the same response as observed with 1 mM D-Ala alone (not shown). This confirms that the current response is effectively due to RgDAAO activity.

2. Incubate the suspension for 30 min at room temperature in a sonicator bath and centrifuge the sample at 3,200×*g* for 30 min; recover the aqueous phase.
3. Centrifuge at 27,000×*g* for 1 h; recover the resulting surnatant for the biosensor analyses. The best measurements are performed by adding an enzyme solution containing similar amounts (in terms of enzymatic units) of the M213G and T60A/Q144R/K152E RgDAAOs into the working electrochemical cell; when the difference in signal between the working and the reference electrode reaches a stable value, record the current value.
4. Calculate the total D-amino acid content in the cheese sample from the recorded amperometric current by using the calibration curve obtained using the D-Ala standard solutions and the enzyme mixture made up of M213G and T60A/Q144R/K152E RgDAAO variants (an example of the current traces obtained from a Grana Padano sample is depicted in Fig. 3), see Note 9.

3.3. Determination of D-Serine Content in the Central Nervous System by a DAAO-Based Microbiosensor

D-Serine is an endogenous ligand for *N*-methyl-D-aspartate (NMDA) receptors, and alterations in its concentration have been related to several brain disorders, especially schizophrenia (21). It represents therefore an important target neuromodulator for the pharmaceutical industry. To monitor D-serine levels *in vivo*, a specific microbiosensor has been developed (20). Here, a platinum microelectrode (25 μm×150 μm) is covered with a membrane of poly-*m*-phenylenediamine (PPD); on the top of the membrane a

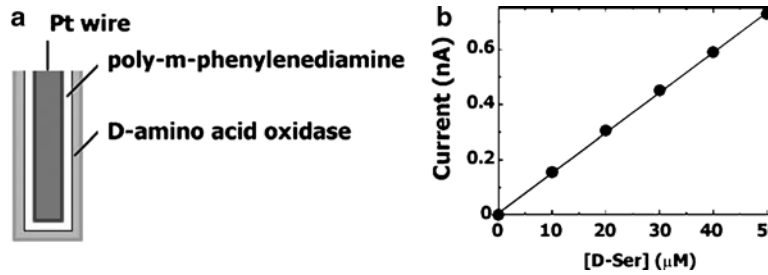

Fig. 4. Design of the RgDAAO microbiosensor for D-Ser detection *in vivo* (20). (**a**) Schematic representation of the microbiosensor: a platinum wire is covered with a layer of poly-*m*-phenylenediamine (PPD) and with the wild-type RgDAAO biocatalyst. (**b**) Calibration curve of a microbiosensor in the 0–50 μM D-Ser concentration range: the oxidation current shows a linear dependence on the D-Ser concentration.

layer of recombinant purified wild-type RgDAAO is deposited by dipping the Pt tip of the electrodes in the enzyme solution (Fig. 4a). The PPD membrane forms a steric barrier that allows H_2O_2 diffusion but blocks larger interfering molecules. The RgDAAO microbiosensors manufactured from a PPD-covered Pt wire, in fact, showed a 97–99% reduction in the interfering responses produced by endogenous oxidating molecules (20); by contrast D-Ser detection is only reduced by 12%. The RgDAAO microbiosensors and control biosensors used for *in vivo* experiments were covered with an additional Naflon membrane to protect the enzyme layer during penetration in the brain. This additional membrane did not change the sensitivity or the response time of the electrodes (see Note 10). D-Ser is detected by a two-step process: (1) RgDAAO catalyzes the oxidative deamination of the D-amino acids present in the proximity of the electrode (i.e., D-Ser is converted into hydroxypyruvate), generating equimolar amounts of H_2O_2 and (2) H_2O_2 is oxidized at the surface of a platinum wire connected to a patch-clamp amplifier. The resulting H_2O_2 oxidation current corresponds to the D-Ser concentration in the biosensor's microenvironment (Fig. 4b).

The *in vivo* experiments are performed as follows.

1. First calibrate the RgDAAO microbiosensor with D-Ser standard solution. The electrodes respond to changes in D-Ser concentration with an increase in the recorded oxidation current (see Note 11). The response time, defined as the duration of the signal increase by between 10 and 90%, is ~2 s.
2. Subsequently calibrate the microbiosensor in an artificial extracellular solution that closely resembles the ionic composition of rat cerebrospinal fluid. In this medium, the sensitivity of the electrodes are unchanged compared to PBS.
3. The RgDAAO microbiosensor is then implanted in the frontal cortex of anesthetized rats (81 mm lateral from the midline,

3 mm anterior from the Bregma, and 1.5 mm ventral from the Dura), side by side (0.5 mm) with a control biosensor (covered with BSA), see Note 12.

4. Wait for at least 30 min to allow currents to stabilize, then start recording them for ≥1 h. A difference in the stabilized background currents between the control and the RgDAAO microbiosensors should be observed (i.e., ~47 pA). This difference should be minimal in calibration tests made in PBS or in the artificial extracellular medium (i.e. 2–3 pA).
5. Provided that O_2 is not limiting, the relationship between the amplitude of the step in oxidation current detected by the microbiosensor and D-Ser concentration can be approximated by a Michaelis–Menten equation:

$$C = \frac{C_{\max}[D\text{-Ser}]}{K_{\mathrm{m,app}} + [D\text{-Ser}]}, \quad (1)$$

where C is the oxidation current and $C_{\max}$ and $K_{\mathrm{m,app}}$ are analogous to the classical $V_{\max}$ and K_{m} kinetic parameters as used for the free enzyme.

6. Calculate the D-Ser concentration using Eq. 1 and the calibration curve obtained with a different D-Ser standard solution (Fig. 4b). See Notes 13 and 14.

4. Notes

Midaspro Biosensor

1. With this calibration procedure, the amperometric signals from different electrochemical cells as well as those from different measurement sessions can be compared.
2. The main drawback of this system is represented by the operational stability. A good response reproducibility is obtained by washing the electrode with sodium hypochloride after each measurement. This entails adding a new aliquot of enzyme for each determination: however, only a very limited amount of RgDAAO is used for each single measurement.
3. The system was also used to estimate the overall content of neutral-basic D-amino acids in milk samples (maintained for 1–30 days at 4°C). The recovery of 1 mM D-Ala added to the crude milk samples is almost complete and no interference at an applied potential of +400 mV is detected (18). D-Amino acid concentration is below the detection limit in fresh milk, but increases with aging to reach a total concentration of 49 μM after 1 month at 4°C. These data are in good agreement

with the values reported in the literature and determined using chromatographic methods (~45 μM) (22).

4. An intense anodic signal is detected after adding the milk sample in the electrochemical cell; however, the amperometric trace returns to the initial value in ~15 min.

Specialities Amperometric Biosensor

5. The biosensor shows a well-defined and quick response after adding wild-type RgDAAO to a 1-mM D-Ala solution in the working cell. In the absence of the enzyme, no amperometric response is observed after adding up to 10 mM H_2O_2 and/or 1 mM D-Ala or pyruvate, indicating that the current response is not due to H_2O_2 or D-amino acid/α-keto acid detection (17). These results indicate that the electrons produced by the enzymatic reaction are directly transferred to the electrode: this device belongs to the third-generation group of biosensors (23).
6. The decrease in apparent K_m values for the D-amino acid offers an important advantage of using immobilized RgDAAO for biosensor-based measurements because of partitioning effects. This effect is especially evident for D-amino acids that bind weakly to DAAO, e.g., K_m for D-Pro is 21.5 and 5.6 mM and K_m for D-Val is 18.9 and 1.3 mM for free and immobilized RgDAAO, respectively (24).
7. The effect of the substrate composition on the amperometric response was evaluated using various immobilized RgDAAO variants and D-amino acid solutions (0.5 mM final concentration) containing different ratios of D-Ala (0–0.5 mM), D-Glu (0–0.4 mM), D-Lys (0–0.4 mM), D-Gln (0–0.16 mM), and D-Met (0–0.1 mM) (17).
8. The protocol for determining D-amino acids based on RgDAAO variants is simple, rapid (~10–15 min), and reliable. The easy, fast, and inexpensive production procedure (the overall cost is ~0.5€/electrode) only requires a single screen-printed electrode for each amperometric analysis ("one-shot" disposable device), with no need to regenerate the electrode surface.
9. The RgDAAO-based device was also successfully used to measure the total D-amino acid content in cheese samples. The amount of D-amino acids determined in Grana Padano cheese samples (~6.1 mM corresponding to 6.7 mg D-amino acid per gram of cheese sample) (17) is in fairly good agreement with the value reported in the literature (10, 11). Interestingly, such a final D-amino acid concentration contains 54% D-Glu, 29% D-Asp, and 17% D-Ala, showing that the RgDAAO-based biosensor is a powerful analytical system even on complex matrices.

This electrochemical method compares favorably with standard methods for overall D-amino acid determination.

Microbiosensor

10. All microbiosensors were tested for the detection of serotonin (20 μM in PBS), D-Ser (1 μM in PBS), and H_2O_2 (1 μM in PBS) before use. Only the electrodes showing more than 7 pA/1 μM D-Ser and less than 4 pA/20 μM serotonin were used to determine D-Ser concentrations.
11. *In vitro* calibrations were performed in standard solutions prepared with PBS. The reference electrode was a chlorided silver wire placed directly into the recording chamber. Recordings were made under constant potential amperometry at +500 mV vs. Ag/AgCl (reference electrode).
12. Implanting the device in the CNS produced a small decrease in sensitivity (13.2%). This decrease is common to virtually all microbiosensors and generally occurs during the first 15 min following insertion into the brain.
13. The intraperitoneal injection of 1 g D-Ser/kg body weight induces a steady increase in oxidation current at the RgDAAO microbiosensor, but not at the control sensor, indicating that the electrochemical signal detected by the microbiosensor is specific for D-Ser (20). This increase in electrochemical signal probably arises from diffusion of D-Ser across the blood–brain barrier.
14. For detecting D-Ser *in vivo* increased selectivity, higher sensitivity, and miniaturization are required to allow implantation in order to implant the device in the CNS of small laboratory animals. These technical challenges have been overcome through: (1) the use of RgDAAO, a very active and selective enzyme; (2) the development of micrometric platinum wire electrodes for H_2O_2 monitoring; and (3) the use of a highly selective PPD layer to block the nonspecific oxidation of endogenous molecules.

Acknowledgments

The work reported in this paper was supported by grants from Fondo di Ateneo per la Ricerca (University of Insubria) to S. Sacchi and L. Pollegioni and from Fondazione Cariplo and Regione Lombardia (bando Cooperazioni Scientifiche Internazionali) to L. Pollegioni. We thank Carlo Rossetti, Jean-Pierre Mothet, and Stéphane Marinesco who over the years have actively contributed to the evolution of DAAO-based biosensors.

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