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A biosensor for all D-amino acids using evolved D-amino acid oxidase

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ABSTRACT

Determination of the D-amino acid content in foods and in biological samples is a very important task. In order to achieve this goal we developed a biosensor employing the flavoenzyme D-amino acid oxidase from the yeast *Rhodotorula gracilis*. To produce a device in which the D-amino acid composition does not alter the results, both the wild-type and a number of mutants obtained by rational design and directed evolution approaches were used. An analysis of D-amino acid oxidase mutants activity on D-amino acid mixtures containing various ratios of neutral, acidic, and basic substrates identified the Amberzyme-immobilized T60A/Q144R/K152E and M213G mutants as the best choice: their response shows an only limited dependence on the solution composition when at least 20% of the D-amino acid is made up of D-alanine (standard error is ~5–9%). This is the first report, to our knowledge, demonstrating that the entire D-amino acid content can be determined by using a screen-printed electrode amperometric biosensor, with a detection limit of 0.25 mM and a mean response time of 10–15 min. The D-amino acid assay based on *R. gracilis* DAAO-biosensor is inexpensive, simple to perform, and rapid: the D-amino acid concentration of a variety of biological samples can be investigated using this assay.

1. Introduction

In recent years it has become an important goal to determine and quantify D-amino acids in biological samples. The occurrence of D-amino acids in food is normally associated with a decrease in protein digestibility, thus affecting the bio-availability of essential amino acids and ultimately impairing the nutritional value of food (Friedman, 1999). Both the concentration of D-amino acids and the D/(D + L) ratio have been proposed as indexes for assessing food quality (and identifying food adulteration) and as reliable molecular markers of ripening (Friedman, 1999; Marchelli et al., 1997).

The D-amino acid concentration in food often depends on the original D-amino acid content of 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 (Friedman, 1999). Anyway, D-amino acid content can also be related to bacterial contamination. The bacterial cell wall consists of peptidoglycans and polysaccharides cross-linked with peptides in which D-Ala is the central molecule and D-Glu is also present; therefore, the D-Ala and D-Glu content can be used to measure bacterial contamination (Gandolfi et al., 1992). Significant amounts of free D-Ala were found in juices affected by bacterial growth and the content of free D-Ala in milk samples increases significantly when milk is stored at 4 °C for 1 month. Several D-amino acids have also been detected in fermented products, where they can arise from "natural" processes (D-Ala, D-Gln, and D-Asp are abundant in cheese, yoghurt, wine, vinegar, etc.) (Friedman, 1999; Marchelli et al., 2006). 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, and the racemization degree of L- and D-isomers can be used to estimate the sample age (Marchelli et al., 1997, 2006). In addition to these specific cases, all the D-isomers of amino acids can be found in foods as a consequence of adulteration, such as in the addition of hydrolyzed proteins to mask low nutrient content, or of technological processes (e.g., significant amounts of D-Asp are present in various kinds of baby formula as a result of the racemization processes of the L-isomer after spray drying and sterilization) (Payan et al., 1985; Marchelli et al., 2006). Diet then exposes mammals to the D-amino acids present in food impairing the nutritional quality of food and arising safety concerns (e.g., exposition to high doses of D-Ser was reported to give nephrotoxic effects in rats) (Pollegioni et al., 2007). For these reasons, the determination of the overall D-amino acid content constitutes an appropriated analytical parameter.

Generally, efficient enantiomeric separations and analyses using high-performance liquid chromatography, gas chromatography, or capillary electrophoresis are performed to determine the D-amino acid content in food, beverages, and biological samples (Friedman, 1999; Payan et al., 1985; Warnke and Armstrong, 2006). Although

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these methods are very sensitive, reliable, and reproducible, they are time-consuming, highly specialized, and expensive and they are often too slow for the food industry (Friedman, 1999). For routine analysis and quality control measurements simple and easily applicable analytical methods are needed: biosensors can often satisfy these requirements.

D-Amino acid oxidases (EC 1.4.3.3, DAAO) are FAD-containing flavoenzymes that oxidize the D-isomers of amino acids with absolute stereospecificity according to the general reaction (Pollegioni et al., 2007):

$$\text{D-Amino acid} + O_2 + H_2O \rightarrow \alpha\text{-ketoacid} + H_2O_2 + NH_3 \quad (1)$$

In past years, many research groups have proposed enzyme sensors that specifically detect D-amino acids by determining the hydrogen peroxide content produced by the reaction catalyzed by DAAO from pig kidney; for a review see Pollegioni et al. (2008). However, application of these sensors was limited by the low specific activity of the mammalian enzyme and the inactivity on acidic D-amino acids (Pollegioni et al., 2007). The first problem was overcome by using DAAO from the yeast *Rhodotorula gracilis* (RgDAAO) (Sacchi et al., 1998), which exhibits a very high turnover number, a broad substrate specificity, and a tight binding with the FAD cofactor (Pollegioni et al., 2007). The second one was recently solved by using two different protein-engineering approaches with which RgDAAO variants could be isolated with an enlarged substrate specificity (and with a remarkably improved catalytic efficiency on the acidic and the basic substrates, as well as on unnatural D-amino acids) (Sacchi et al., 2002, 2004; Caligiuri et al., 2006).

The aim of the present work is to apply RgDAAO mutants obtained both by rational design and directed evolution approaches in a pilot biosensor to rapidly determine the total content of D-amino acids in biological samples. We report on the set-up of a simple "one-shot" (disposable) device characterized by low cost and ease of use, properties that represent major advantages of our enzymatic biosensor.

2. Materials and methods

2.1. Expression and purification of enzymes

Recombinant wild-type, T60A/Q144R/K152E, Q144R (Sacchi et al., 2004), M213R (Sacchi et al., 2002), and M213G (Caligiuri et al., 2006) mutants of RgDAAO were expressed in BL21(DE3)pLysS *E. coli* cells using the pT7-HisDAAO expression vector and then purified by Hitrap Chelating chromatography (GE Healthcare) (all of them contain an His-tag at the N-terminal end). Moreover, recombinant Y238R, Q339E, and M213E RgDAAO mutants (Boselli et al., 2007) were prepared as previously reported for the wild-type protein (Molla et al., 1998). The protein concentration of the purified enzymes was estimated using the extinction coefficient ($12.6\,mM^{-1}\,cm^{-1}$ for wild-type DAAO at 455 nm) (Molla et al., 1998).

2.2. Activity assay and kinetic measurements

DAAO activity was assayed with an oxygen electrode at air saturation (0.253 mM O_2) and 25 °C, using 28 mM D-Ala as substrate in 75 mM sodium pyrophosphate buffer, pH 8.5 (Molla et al., 1998). One DAAO unit is defined as the amount of enzyme that converts 1 μmol of D-amino acid per minute at 25 °C. The initial reaction rates at different substrate concentrations were used to calculate the kinetic parameters employing the KaleidaGraph software (Synergy Software). The substrate specificity was investigated by means of the same polarographic assay, employing different concentrations of various D-amino acids as substrate.

In order to identify the most suitable DAAO mutants to quantify the total content of D-amino acids, we measured the initial rate of oxygen consumption using solutions of D-amino acids (10 mM final concentration) containing different ratios of D-Ala (0–10 mM), D-Glu (0–8 mM), D-Lys (0–8 mM), D-Gln (0–3.3 mM), and D-Met (0–2 mM) and 0.05 units of each RgDAAO mutant enzyme. The theoretical activity values on these amino acid mixtures were calculated using the multi-substrate Michaelis–Menten Eq. (2):

$$V_{tot} = \frac{V_{max,D\text{-}Ala}\cdot[D\text{-}Ala]}{[D\text{-}Ala]+K_{m,D\text{-}Ala}} + \frac{V_{max,D\text{-}Glu}\cdot[D\text{-}Glu]}{[D\text{-}Glu]+K_{m,D\text{-}Glu}} + \frac{V_{max,D\text{-}Lys}\cdot[D\text{-}Lys]}{[D\text{-}Lys]+K_{m,D\text{-}Lys}} + \frac{V_{max,D\text{-}Gln}\cdot[D\text{-}Gln]}{[D\text{-}Gln]+K_{m,D\text{-}Gln}} + \frac{V_{max,D\text{-}Met}\cdot[D\text{-}Met]}{[D\text{-}Met]+K_{m,D\text{-}Met}} \quad (2)$$

and the apparent kinetic parameters determined for these RgDAAO enzyme variants for each D-amino acid.

In order to identify competitive inhibition effects due to the presence of alternative substrates (e.g., A and B) in a complicated situation in which the products are identical for the assay method used (as in this case), we calculated the theoretical activity values by using the steady-state equation proposed by (Segel, 1993):

$$V_{tot} = \frac{V_{max,A}\cdot[A]/K_{m,A} + V_{max,B}\cdot[B]/K_{m,B}}{1+[A]/K_{m,A}+1+[B]/K_{m,B}} \quad \text{or}$$

$$V_{tot} = \frac{V_{max,A}\cdot[A]}{K_{m,A}\cdot(1+[B]/K_{m,B})+[A]} + \frac{V_{max,B}\cdot[B]}{K_{m,B}\cdot(1+[A]/K_{m,A})+[B]} \quad (3)$$

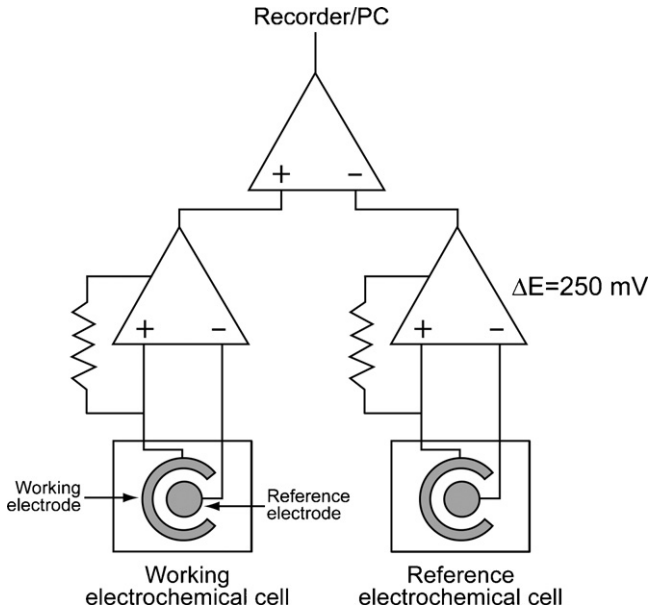
Thus, one enzyme that accepts two substrates will give results similar to two enzymes each of which is specific for one substrate but is competitively inhibited by the other's substrate. The V_{tot} observed in the presence of any given mixture of A and B will be the same as the V observed with A or B alone at the same total specific concentration only if A and B are equally acceptable substrates (i.e., same V_{max} and K_m parameters).

2.3. Enzyme immobilization

Purified RgDAAO mutants were covalently immobilized on an Amberzyme Oxirane support (Rohm and Haas Advanced Biosciences) by a coupling procedure involving protein-free amino groups. The dry matrix (2.5 g) was washed at room temperature for 15 min with 400 mM potassium phosphate, pH 4.6; the suspension was dried, 250 units of enzyme were added, and then it was incubated under constant stirring for 7 h at room temperature. The matrix was then packed into a column and washed with 100 mM potassium phosphate, pH 8.0. The immobilized enzyme activity was determined by a polarographic method as described above using a weighted amount of matrix suspended ($100\,mg\,ml^{-1}$) in 100 mM potassium phosphate buffer, pH 8.0.

2.4. Biosensor: electrochemical cells and amperometric procedures

Electrochemical experiments were carried out in a home-made biosensor consisting of two plastic cells containing a working electrode (graphite disk, 28 mm in diameter) and a reference electrode (Ag/AgCl). Electrochemical cells were fabricated by using a screen-printing process (Specialities s.r.l.); the amperometric biosensor was connected to a home-made data acquisition and processing system (see Scheme 1). This device is an improvement of the original biosensor developed using RgDAAO that was based on H_2O_2 detection (Sacchi et al., 1998). The new biosensor relies on the most intimate contact between the biocatalyst and electrode surface and the response is the difference in signals between the working and the reference electrode that differ for the presence of the enzyme



Scheme 1. Schematic representation of the biosensor for amperometric determination of D-amino acids. The output is the difference in signal between the working cell (containing the enzyme) and the reference cell.

in the previous. To determine the optimal electrode potential we measured the signal given by 1 mM D-Ala at increasing applied potentials (from 150 to 800 mV). A value of 250 mV was chosen as the measuring potential since the optimal amplitude of the signal was coupled to a low operating potential that reduced interference (in particular that from the electrochemically active species present in many food matrices).

Amperometric measurements were carried out in the electrochemical cells containing 3 ml of D-amino acid solution in 100 mM potassium phosphate buffer, pH 7.0, at room temperature. Then, a small amount of different immobilized RgDAAO variants (0.5–4.0 units) was added in the working cell and the current was recorded. A calibration curve was obtained using a standard solution of D-Ala (in the 0.25–5 mM final concentration range). To detect D-amino acids from complex samples (such as cheese samples, see below), RgDAAO was added when the difference in signal between the working and the reference electrode reached a stable value, i.e. after few minutes from sample addition. The effect of the substrate composition on the amperometric response was evaluated using different 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).

Data are expressed as mean ± standard deviation (S.D.) except for the mean values on different D-amino acid mixtures, where we used mean ± S.E. of the mean (S.E.M.). Statistical tests were performed using Kaleidagraph (Synergy Software).

2.5. Biosensor: test on real samples

Finely grated Grana Padano cheese samples were suspended in 100 mM potassium phosphate buffer, pH 7.0 at a final concentration of 0.1 g ml^{-1}. The resulting suspension was sonicated for 30 min and centrifuged at 3200 × *g* for 30 min. The aqueous phase was newly centrifuged at 27,000 × *g* for 1 h and the resulting supernatant was used for the biosensor analyses. For sake of comparison, the amount of D-amino acids in cheese samples was also determined spectrophotometrically following their enzymatic conversion into the corresponding α-keto acids and reaction with 2,4-dinitrophenylhydrazine (Caldinelli et al., 2006).

3. Results

3.1. Selection of RgDAAO variants active on all D-amino acids

The dependence of the activity value on the substrate composition for the RgDAAO mutants obtained by site-directed mutagenesis (M213R, M213G, M213E, Y238R and Q339E DAAOs) or by error-prone PCR (Q144R and T60A/Q144R/K152E DAAOs) was assessed by measuring the initial velocity of the enzyme reaction on mixtures of D-amino acids (10 mM final concentration) containing different ratios of D-Ala, D-Glu, D-Lys, D-Gln, and D-Met as substrate (Fig. 1). When compared with the value measured on 10 mM D-Ala (taken as 100%), the activity of the M213R and M213G RgDAAO mutants was appreciably lower on mixtures not containing D-Glu (e.g., a 30 and 40% of the value determined on 10 mM D-Ala was determined on mixture 6, respectively). On the other hand, only RgDAAO mutants at position 213 show activity that is similar to the reference value in the absence of D-Ala in the reaction mixture (mixture 8), as well as a significantly high response in the presence of all five D-amino acids used (mixture 7). Instead, the activity of the Q339E DAAO strongly decreases in the absence of D-Ala (mixture 8).

Comparing the average of the relative activity values measured for each DAAO mutant on mixtures 1–8, the enzymes containing the Q144R substitution exhibit the lowest variability of response as a function of the D-amino acid composition (Fig. 2): a mean relative activity value corresponding to ∼75% (S.D. = 20%) of the value determined on 10 mM D-Ala was measured. The DAAO mutants at position M213 showed an average response that is very close to the value determined on 10 mM D-Ala, but for the M213E and M213R DAAO mutants a significant variability that is dependent on the substrate composition was also evident (Fig. 2).

3.2. Theoretical analysis

In order to estimate the concentration of D-amino acids from the DAAO activity values that were measured, we verified the correlation between our experimental results and the theoretical values that can be calculated using the Michaelis–Menten equation. The apparent kinetic parameters at 21% oxygen saturation ($V_{max,app}$ and $K_{m,app}$ values) were determined for the wild-type and the best RgDAAO mutants identified from the previous analysis (M213R, M213G and T60A/Q144R/K152E, see above and Figs. 1 and 2) on D-Ala, D-Glu, D-Lys, D-Gln, and D-Met as substrates: for all the RgDAAO mutants, the kinetic parameters show a strong dependence on the substrate employed (Table 1). The Michaelis–Menten equation and the kinetic parameters were then used to calculate the theoretical activity values for each RgDAAO variant in the presence of different ratios of three D-amino acids: a neutral, a basic, and an acidic D-amino acid ($V_{tot} = V_{o,D\text{-}Ala} + V_{o,D\text{-}Glu} + V_{o,D\text{-}Lys}$), Eq. (2). As depicted in the three-dimensional plot in Fig. 3 (the value on 10 mM D-Ala is taken as 100%), the theoretical activity value for the T60A/Q144R/K152E RgDAAO mutant showed the lowest dependence on the amino acid composition when the D-Ala concentration is ≥20% of the mixture composition (≥2 mM). By contrast, the M213R RgDAAO was not active on basic D-amino acids and its catalytic efficiency on neutral D-amino acids suffered a dramatic decrease (see Table 1). Consequently, as shown in the three-dimensional plot, the theoretical activity of M213R RgDAAO exhibited a stronger dependence on the amino acid composition.

Comparing the experimental data obtained by the polarographic assay (Fig. 1) with the theoretical values (Fig. 3) a good corre-

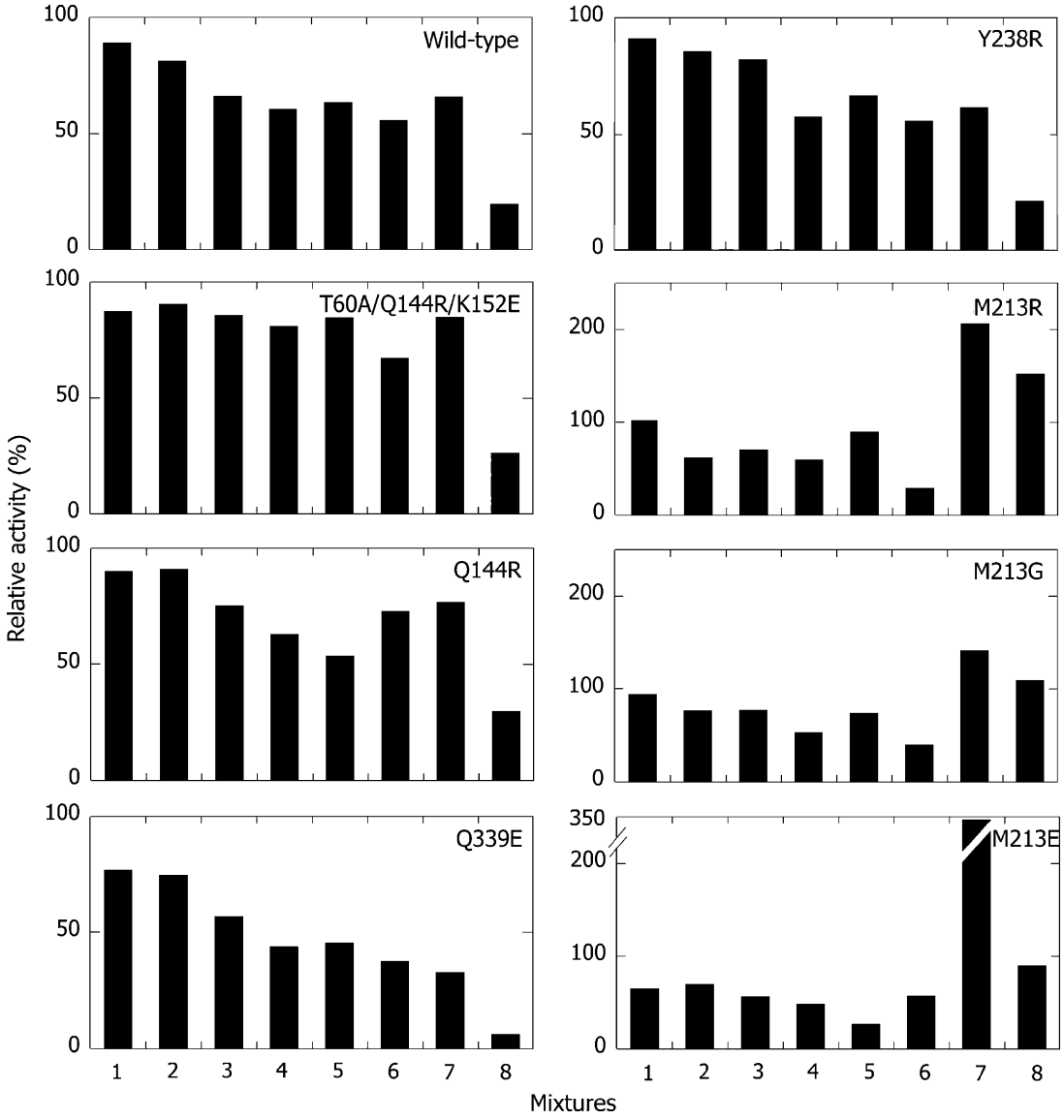


Fig. 1. Relative activity determined for wild-type and mutants (T60A/Q144R/K152E, Q144R, Q339E, Y238R, M213R, M213G, M213E) of RgDAAO on 10 mM D-amino acid solutions containing different ratios of D-Ala/D-Glu/D-Lys/D-Gln/D-Met. The activity was determined by the oxygen consumption assay, at 25 °C and pH 8.5. The value measured on 10 mM D-Ala was taken as 100%. Mixture composition: (1) 5 mM D-Ala, 5 mM D-Glu; (2) 5 mM D-Ala, 5 mM D-Lys; (3) 3.3 mM D-Ala, 3.3 mM D-Glu, 3.3 mM D-Lys; (4) 2 mM D-Ala, 4 mM D-Glu, 4 mM D-Lys; (5) 2 mM D-Ala, 8 mM D-Glu; 6) 2 mM D-Ala, 8 mM D-Lys; 7) 2 mM D-Ala, 2 mM D-Glu, 2 mM D-Lys, 2 mM D-Gln, 2 mM D-Met; (8) 3.3 mM D-Glu, 3.3 mM D-Lys, 3.3 mM D-Gln.

lation was evident for mixtures 1–5 (see Table 2). On the other hand, the calculated V_{tot} values determined on mixtures 6–8 for the employed mutant RgDAAOs using the Michaelis–Menten equation (Eq. (2)) differed significantly from the experimental values (Table 2): the most significant changes were observed for the wild-type and M213G RgDAAOs. In fact, when a single enzyme is active on different substrates, and these are simultaneously present, each substrate could act as a competitive inhibitor with respect to the other (Segel, 1993). A better correlation between the theoretical values and the experimental results on mixtures 6–8 was obtained when the equation of an ordinary competitive inhibition process was used (Eq. (3), see last column in Table 2), thus indicating the presence of inhibitory effects on complex mixtures that could drop the estimated amount of D-amino acids. Using the competitive inhibition equation, and with the only exception of mixture 8 which does not contain D-Ala, the T60A/Q144R/K152E DAAO mutant showed the best correlation with the experimental values.

3.3. Set-up of a DAAO biosensor for all D-amino acids

The reaction of RgDAAO on D-amino acids can be followed by different assays, such as by the use of a biosensor: for a recent review see Caldinelli et al. (2006). In order to clarify the communication between RgDAAO and the electrode in our biosensor, various control experiments were performed. The biosensor we developed shows a well-defined and quick response following the addition of wild-type RgDAAO (in the working cell, see Scheme 1) to a 1 mM D-Ala solution. In the absence of the enzyme, no amperometric response was obtained after adding up to 10 mM H_2O_2 and/or 1 mM D-Ala or pyruvate, indicating that the current response was not due to H_2O_2 or D-amino acid/α-keto acid detection. 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 (Chaplin and Bucke, 1990). This conclusion was demonstrated by introduc-

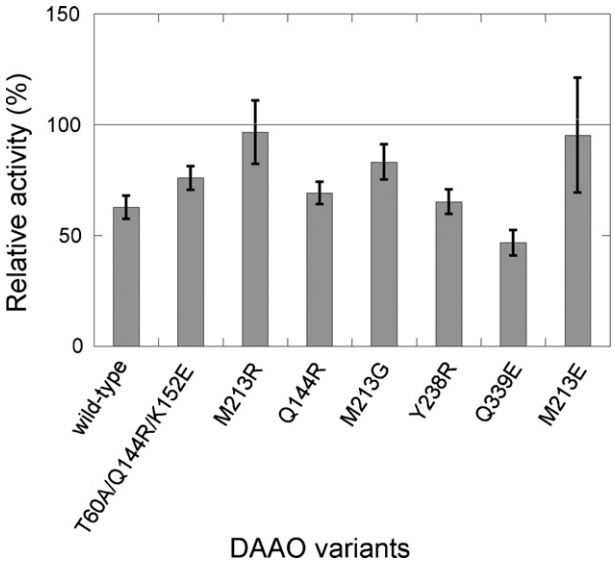


Fig. 2. Average of the activity values determined for wild-type and mutants of RgDAAO on the D-amino acid solutions used in Fig. 1. Bars indicate mean ± S.E.M. (n = 24) for the determination on the eight different D-amino acids mixtures as in Fig. 1.

ing the enzyme in the working cell separately from the solution by a dialysis membrane, which allows to small molecules (such as the substrate/product) to freely diffuse but prevents a direct contact between the electrode and the enzyme. In this case, and although the spectrophotometric analytical method for D-amino acids detection showed that >80% of substrate (1 mM D-Ala) was converted after 10 min, no amperometric signal was recorded.

Concerning the set up of the biosensor, a higher response was observed in a static solution than under stirring using the free form of the enzyme, with a coefficient of variation (CV = S.D./mean, n = 6) corresponding to 28%. Analogously, the enzyme immobilized on the Amberzyme Oxirane support also showed a higher amperometric response in still cells compared to that in a stirred solution. At increasing speed of stirring a decrease of the amperometric response was observed (at 300 rpm only 10% of the value measured under static conditions is recorded) in concert to an increase of signal noise. We propose that it is due to the lower amount of enzyme that can efficiently contact the electrode surface for direct electron transfer. Importantly, a comparatively higher response was recorded with the immobilized RgDAAO than when using the free enzyme form (up to 1.5-fold), which was also coupled with good repeatability (CV ≤ 15%, n = 6). A further main advantage to use RgDAAO in the immobilized form is given by the decrease of apparent K_m values because of partitioning effects. This effect is more evident for D-amino acids that are not good substrates for 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 (Pilone et al.,

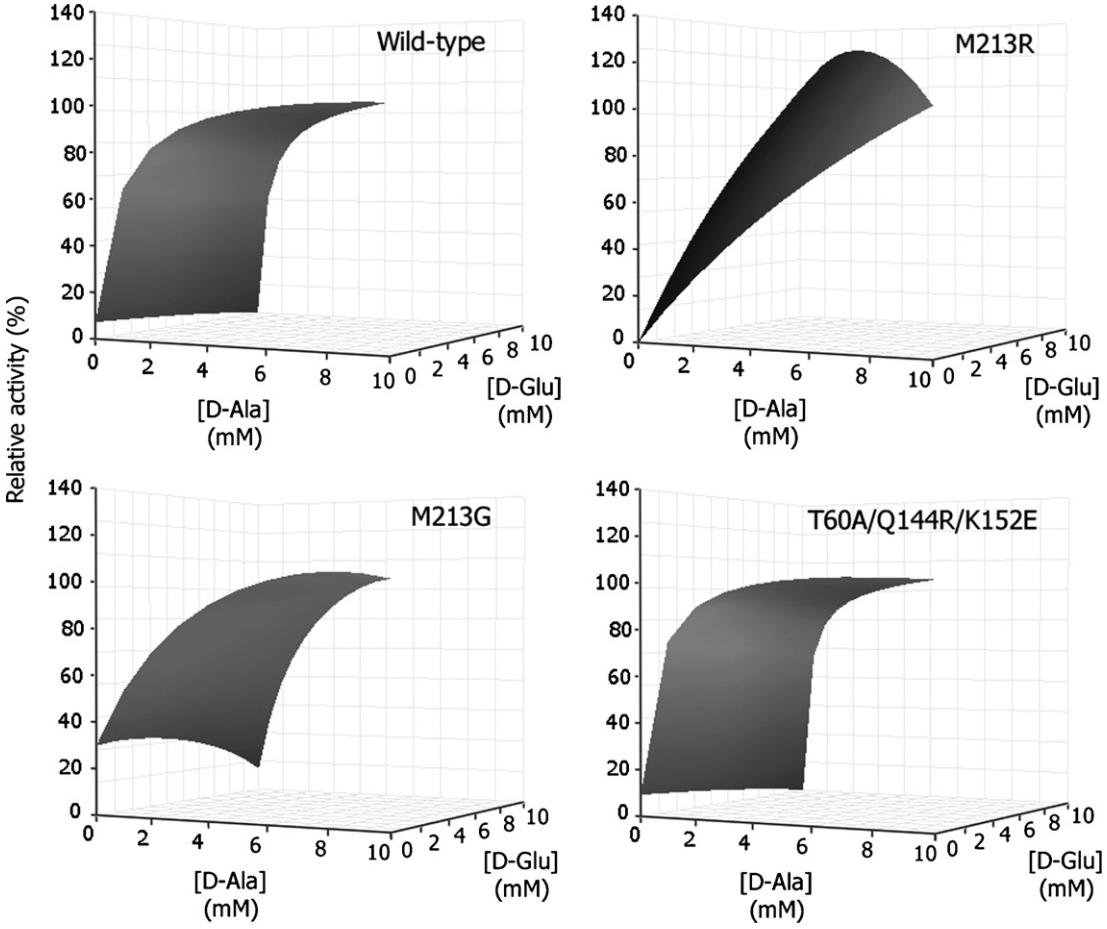


Fig. 3. Three-dimensional plots showing the theoretical dependence of the activity value determined for wild-type, M213R, M213G and T60A/Q144R/K152E RgDAAOs on the composition of a 10 mM D-amino acid solution made up of D-Ala, D-Glu and D-Lys. The concentration of this latter component in the assay solution is the value obtained after subtracting the D-Ala and D-Glu values from the final concentration (not shown).

1992). Importantly, this effect also confers a lower dependence of the enzymatic activity on substrate composition. Altogether, these results suggest that the use of RgDAAO in the immobilized form and in a static cell represents the best set-up for the biosensor.

Under optimized working conditions, we calibrated the biosensor containing 2.5 units of immobilized wild-type RgDAAO, measuring the amperometric response following the addition of D-Ala standard solutions (0.25–5.0 mM range). The current values detected after 10 min were plotted as a function of substrate concentration and fitted by means of a classic Michaelis–Menten equation (not shown). The detection limit is 0.25 mM D-Ala (signal/noise = 3). The best RgDAAO mutants, as identified by the kinetic measurements on different mixtures of amino acids (see above), were then used in the immobilized form in the biosensor to measure 0.5 mM solutions of D-amino acids containing different ratios of D-Ala, D-Glu, D-Lys, D-Gln, and D-Met. The highest response, compared to the one on 0.5 mM D-Ala taken as 100%, was measured for the T60A/Q144R/K152E and M213G RgDAAO mutants (Fig. 4). This and the low dependence of the response on the substrate composition (S.E.M. = 5% and 9% for M213G and T60A/Q144R/K152E, respectively) identify the latter immobilized RgDAAOs as the best choice for such an application.

3.4. D-Amino acid measurements on real samples

D-Amino acids can act as indicators of both microbial contamination and ripening processes (see Section 1). The performance of our biosensor as a means of monitoring the entire D-amino acid content in foods, such as Grana Padano cheese, was assessed during this study. An increase in signal response proportional to the amount of sample is observed when testing such cheese samples (Fig. 5). By means of the calibration curve an average content of 6.1 ± 0.5 mM D-amino acids was determined, corresponding to ~6.7 D-amino acid per cheese sample (mg g^{-1}). Interestingly, this value is in good agreement with the amount estimated by the alternative 2,4-dinitrophenylhydrazine method (6.0 ± 0.3 mg g^{-1}) and with data in literature based on chromatographic determinations (Marchelli et al., 2006). In order to verify the specificity of the assay,

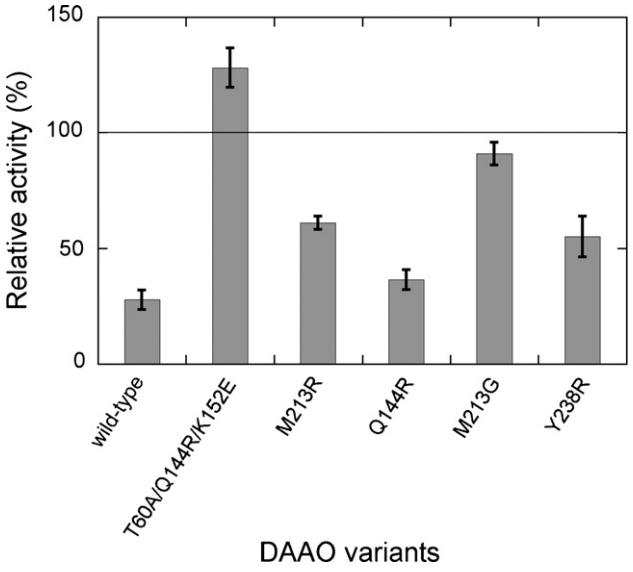


Fig. 4. Average of the amperometric response of the enzymatic biosensors produced using immobilized wild-type and mutants of RgDAAO obtained on eight different 0.5 mM mixtures of D-amino acids (these solutions were prepared by 20-fold dilution of the ones reported in Fig. 1 and Table 2). Data are expressed as mean ± S.E.M. ($n = 24$).

Table 1
Substrate specificity of different RgDAAOs

	D-Ala			D-Glu			D-Lys			D-Gln			D-Met		
	$V_{max,app}$ (U/mg)	$K_{m,app}$ (mM)	$V_{max,app}/K_{m,app}$	$V_{max,app}$ (U/mg)	$K_{m,app}$ (mM)	$V_{max,app}/K_{m,app}$	$V_{max,app}$ (U/mg)	$K_{m,app}$ (mM)	$V_{max,app}/K_{m,app}$	$V_{max,app}$ (U/mg)	$K_{m,app}$ (mM)	$V_{max,app}/K_{m,app}$	$V_{max,app}$ (U/mg)	$K_{m,app}$ (mM)	$V_{max,app}/K_{m,app}$
Wild-type	98 ± 5.0	0.9 ± 0.1	108	1.7 ± 0.1	76 ± 14	0.02	16 ± 0.5	14.6 ± 1.4	1.1	82 ± 0.9	5.4 ± 0.2	15	162 ± 6.0	0.6 ± 0.09	270
M213R	16.6 ± 0.7	18.5 ± 2.0	0.9	20 ± 0.4	21.1 ± 1.2	1.0		b.d.		26 ± 0.4	5.4 ± 0.3	4.8	24.7 ± 0.3	1.2 ± 0.06	21
M213G	19.2 ± 0.3	4.9 ± 0.5	2.3	2.0 ± 0.04	5.1 ± 0.5	0.4	6.9 ± 0.3	8.0 ± 1.0	0.9	67.7 ± 2.7	2.5 ± 0.4	27	70.8 ± 2.6	0.8 ± 0.1	89
T60A/Q144R/K152E	92 ± 2.1	0.6 ± 0.06	153	2.4 ± 0.2	101 ± 14	0.02	21.4 ± 0.5	16.6 ± 1.0	1.3	93 ± 1.8	6.0 ± 0.4	16	100 ± 1.0	0.3 ± 0.01	335

The apparent kinetic parameters were determined for wild-type, M213R, M213G, and T60A/Q144R/K152E RgDAAO mutants on five D-amino acids as substrate using the oxygen assay (at 25 °C, pH 8.5). b.d. = below detection.

Table 2
Comparison of the experimental and theoretical activity values of wild-type, M213R, M213G, and T60A/Q144R/K152E mutants of RgDAAO on different D-amino acids mixtures

Mixture	Enzyme	Experimental value	Theoretical value			
			Michaelis–Menten equation		competitive inhibition equation	
		(U/mg protein)	(U/mg protein)	(%)	(U/mg protein)	(%)
1	Wild-type	83.5	83.2	100	82.2	95
	M213R	6.9	7.4	107	6.1	88
	M213G	10.2	10.7	105	7.2	71
	T60A/Q144R/K152E	68.6	82.3	120	81.7	119
2	Wild-type	76.3	87.1	114	79.5	104
	M213R	4.2	3.5	83	3.5	83
	M213G	8.3	12.4	149	9.0	108
	T60A/Q144R/K152E	70.9	87.1	123	80.2	113
3	Wild-type	62.0	80.0	129	73.5	118
	M213R	4.8	5.2	108	4.6	96
	M213G	8.4	10.5	125	6.3	75
	T60A/Q144R/K152E	67.2	81.5	121	75.8	113
4	Wild-type	56.8	71.1	125	62.6	110
	M213R	4.0	4.8	120	4.3	108
	M213G	5.7	8.7	153	4.8	84
	T60A/Q144R/K152E	63.6	75.0	118	67.6	106
5	Wild-type	59.6	67.7	114	65.5	110
	M213R	6.0	7.1	118	6.3	105
	M213G	8.0	6.8	85	3.7	46
	T60A/Q144R/K152E	66.3	71.0	107	69.5	105
6	Wild-type	52.3	73.3	140	60.1	115
	M213R	2.0	1.6	80	1.6	80
	M213G	4.3	9.0	209	6.1	142
	T60A/Q144R/K152E	52.8	77.7	147	65.8	125
7	Wild-type	61.8	216.3	350	111.5	180
	M213R	13.9	25.8	186	16.8	121
	M213G	15.3	82.1	537	45.1	295
	T60A/Q144R/K152E	66.5	183.3	276	87.8	132
8	Wild-type	18.4	34.1	185	28.6	155
	M213R	10.3	12.6	122	10.8	105
	M213G	11.8	43.3	367	27.7	235
	T60A/Q144R/K152E	20.8	36.6	176	31.1	150
10 mM D-Ala	Wild-type	93.8	89.9	96	–	–
	M213R	6.8	5.8	85		
	M213G	10.8	12.9	120		
	T60A/Q144R/K152E	78.4	86.8	111		

The activity values were measured on mixtures 1–8 (see legend of Fig. 1) and on 10-mM D-Ala. The theoretical values were calculated using the Michaelis–Menten equation (Eq. (2)) and the competitive inhibition equation in a mixture of alternative substrates (Eq. (3)) and reported as percentage of the corresponding experimental value.

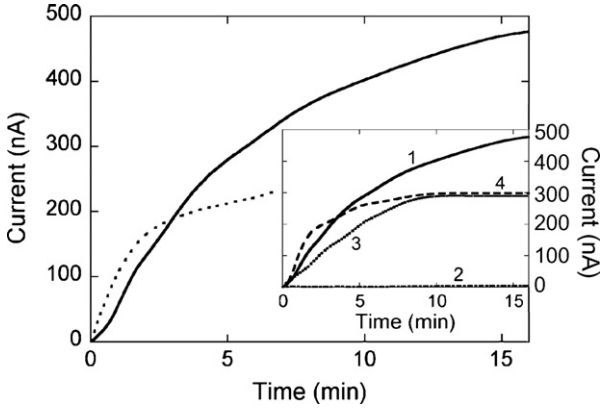


Fig. 5. Response of the RgDAAO biosensor during the detection of D-amino acids in Grana Padano cheese samples. Cheese samples prepared as detailed in Section 2 (—, 2 ml; - - -, 1 ml) were introduced in the 3 ml-cell of the biosensor. *Inset*: the 2 ml Grana Padano sample (—, 1) was treated for 20 min with 1.5 U of RgDAAO before measurement (–·–, 2). Then 1 mM D-Ala (. . ., 3) was added to this sample and after 10 min gave the same response as observed with 1 mM D-Ala alone (– – –, 4).

2 ml of the cheese sample was treated with 1.5 units of RgDAAO before carrying out the biosensor measurement: no response was then observed because the D-amino acids were eliminated by the enzymatic pre-treatment (line 2 in Fig. 5 *inset*). The subsequent addition of 1 mM D-Ala to the cheese sample pre-treated with RgDAAO gave the same response observed with 1 mM D-Ala alone (line 3 in Fig. 5 *inset*).

The overall D-amino acid concentration determined by the RgDAAO biosensor was then validated by measuring the same sample after 1:1 dilution with a solution containing the same concentration of D-Ala. In fact, the response should be significantly different from the original one if the D-amino acid composition of the original solution is outside the region of non-dependence from its composition (see Fig. 3) or if competitive inhibitory effects are active. By using the immobilized T60A/Q144R/K152E RgDAAO mutant, the addition of 0.5 mM D-Ala to an equal volume of 0.5 mM D-amino acid mixtures (see above) resulted in a response value equal to 128 ± 10% of the original one. Significantly, a lower variability of response was obtained using a mixture constituted by the same amount (in terms of enzyme units) of M213G and T60A/Q144R/K152E RgDAAO mutants (91.8 ± 7.2%).

4. Discussion

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 of food or arise from bacterial cell walls. For this reason, quality control is becoming more and more important both for the industry (as a cost factor) and for consumer protection. Therefore, if D-Ala can be considered an indicator of milk contamination (Payan et al., 1985) the presence of other D-amino acids can be specifically related to food processing (e.g., yoghurt contains D-Asp and D-Glu in addition to D-Ala) (Marchelli et al., 1997). On the other hand, the presence of all D-amino acids can be the consequence of adulteration as well as of technological food processing.

For routine food analysis and quality control simple and easily applicable analytical methods are needed: biosensors can often satisfy these requirements. Up to now, practically all the DAAO-based biosensors reported use the enzyme from pig kidney and are therefore characterized by low stability, low turnover number, and inactivity on acidic D-amino acids. A further major drawback of these systems is the different sensitivity to different D-amino acids as substrates (Pollegioni et al., 2007), which implies that it is not possible to determine the total D-amino acid content. As an example, a pig kidney DAAO-based screen-printed amperometric biosensor showed a response varying from 55% to 166% (with D-Arg and D-Val, respectively) of the value determined on D-Phe and was fully inactive on D-Pro (Sarkar et al., 1999).

The biosensor based on a third-generation electrode and RgDAAO mutants responds to all (neutral, acidic and basic) D-amino acids. The M213G and the T60A/Q144R/K152E variants (better if used simultaneously) represent the best RgDAAO mutants for this application. These enzyme mutants show the lowest variability of response as a function of the D-amino acid composition, in particular when the D-Ala concentration is $\geq$20% of the mixture composition and when the enzyme is used in the immobilized form. In addition, the T60A/Q144R/K152E RgDAAO exhibits the best correlation between theoretical activity values on various D-amino acid mixtures and the experimental ones. Due to the additive nature of the individual amino acid responses this device can be used to analyze solutions containing both single amino acid species and complex D-amino acid mixtures. The protocol for determining D-amino acids based on RgDAAO mutants is simple, rapid ($\sim$10–15 min), and reliable. The easy, fast, and inexpensive production procedure (the overall cost is $\sim$0.5 €/electrode) only requires a single screen-printed electrode for each amperometric analysis ("one-shot" disposable device), with no need to regenerate the electrode surface. Because of the high specific activity of RgDAAO, less enzyme is needed (2.5 units = 20 μg) than used in previous biosensors based on mammalian DAAO ($\geq$30 μg) (Váradi et al., 1999). The RgDAAO-based device was successfully used to monitor the total D-amino acid content in a cheese sample: the amount of D-amino acids determined in Grana Padano cheese samples is in fairly good agreement with the value reported in the literature (Marchelli et al., 2006). Interestingly, such a final D-amino acid concentration is constituted by 17% D-Ala, 29% D-Asp, and 54% D-Glu, indicating that the RgDAAO-based biosensor is a powerful analytical tool even on complex matrices. In conclusion, this electrochemical method compares favorably with standard methods (gas chromatography, HPLC or CE techniques) for overall D-amino acid determination.

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