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Medium/High Throughput D-Amino Acid Oxidase Colorimetric Method for Determination of D-Amino Acids. Application for Amino Acid Racemases

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Abstract

Free amino acids detection and quantification in samples still calls for elaborated, powerful and usually high cost and sometimes time-consuming technologies such as gas chromatography, HPLC or highly selective and sensitive column-switching techniques. Here, we undertook to develop a simple, one-step, microtiter plate method to detect and/or quantify most D-amino acids in solutions based on the enzyme D-amino acid oxidase and the generation of stoechiometric amounts of hydrogen peroxide. Inclusion of horseradish peroxidase and OPD allows the determination of D- amino acid concentration through the absorbance of the reaction products as compared to serial dilutions of equimolar amounts of L/D amino acid standards. The methodology is easily adapted for a medium/high throughput analysis of amino acid racemase activities.

Keywords: D-amino acids; Proline racemase; D- amino acid oxidase; amino acid racemases; Alzheimer; aging

Abbreviations: DAAOx: D-amino acid oxidase; HRP: Horse Radish Peroxydase; PYC: Pyrrole-2-carboxylic acid; LOD: Limit of Detection; LOQ: Limit of Quantification; *Tc*PRAC: *Trypanosoma cruzi* Proline Racemase

Introduction

Amino acids are important molecules of life since they constitute the basic structural unit of proteins. The vast majority of natural amino acids can exhibit either a L- or D- form due to their asymmetric carbon. For a long time, L- amino acids were believed to be the only constituents present in eukaryotes whereas D- forms would be present only in prokaryotes (Fisher, 2007, Fujii and Saito, 2004). The presence of amino acid racemases in eukaryotes (Reina-San-Martin et al., 2000; Wolosker et al., 1999), as well as in prokaryotes (Stadtman and Elliott, 1957), enables the active inter-conversion of free L- and D-amino acids. This inter-conversion makes possible a variety of essential cellular metabolic pathways to take place and/or the regulation of intracellular and extracellular amino acid pool as a source of carbon or energy (Chamond et al., 2005).

On the other hand, there is a considerable interest in evaluating the presence and the level of free D-amino acids in samples from food and beverages, since the occurrence of some D-amino acids indicates the presence of contaminant bacterial racemases activities and thus allows the evaluation of fermentation processes. For example, D-Alanine, as well as D-glutamate and D-aspartate are considered as markers for milk fermentation or aging and cheese maturation (Bruckner et al., 1993; Csapo et al., 1995). While D-Alanine and D-aspartate as well as D-Glutamate are in the highest relative amounts in fermented beer, D-Alanine is considered the marker of bacterial contamination of fruit juices and milk. The source of vinegar is established by the analysis of the D- amino acids profile exhibited by the product, which includes the presence of D-Proline, D-Alanine, D-Arginine D-Glutamate and D-Aspartate (Carlavilla et al., 2006). The occurrence of D-amino acids as a result of fermentation of food by contaminant bacteria, makes the detection of D-amino acids a central part of quality control in food industry.

Recent investigations have shown that free D-amino acids play important roles in the physiology of higher organisms. For example, D-Serine was described as a physiologically relevant neuronal modulator (Fuchs et al., 2005). Moreover, D-serine and D-alanine participate in other important functions of the brain, such as learning and nociception, but also in physiopathological processes like schizophrenia and epilepsy (Collingridge, 1987; Fuchs et al., 2005; Palazzo et al., 2002). D-Aspartic acid was shown to be implicated in mammalian ontogeny and differentiation, as well as in modulation of hormonal secretion (Hashimoto et al., 1993; Ishio et al., 1998; Sakai et al., 1998). Interestingly, levels of D-Alanine, D-serine and D-aspartate in the brain and in cerebrospinal fluid have been related to degenerative processes in patients with Alzheimer as compared to healthy individuals (Fisher et al., 1998). Higher levels of D- amino acids are also observed in the serum of individuals with renal diseases and elderly people, as compared to healthy or younger subjects (Bruckner and Hausch, 1993; Fujii, 2005; Nagata et al., 1987; Nagata et al., 1992).

In the present state of the art, D-amino acids are regularly detected and determined by time-consuming physicochemical methods involving HPLC gas chromatography (Erbe and Bruckner, 2000; Patzold and Bruckner, 2006) using chiral stationery phases and High Performance Capillary Electrophoresis (HPCE) (D'Aniello et al., 2000, Erbe and Bruckner, 2000; Patzold and Bruckner, 2006; Pirkle et al., 1984; Thongkhao-On et al., 2004). More complex techniques were also described and call for the use of enantioselective gas or liquid chromatography-tandem mass spectrometry (Bruckner and Westhauser, 2003; Song et al., 2007) or post column LC detection systems for the stereo selective detection of L- and D-amino acids (Casado et al., 2007; Dominguez et al., 1990; Oguri et al., 2005). Less demanding enzymatic methods were developed and some combine microchip-based capillary-zone electrophoresis multi-photon

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excited fluorescence and single laser sources (Gostkowski et al., 1998; Sheeley et al., 2005). Other, straightforward strategies have also been used, such as those dependent on D-amino acid oxidase (Nagata et al., 1985) or more specifically on D-aspartate oxidase (Fisher et al., 1992; Nagata et al., 1985). However, those previously described methods although simple, depend on spectrophotometry or sophisticated biosensor apparatus include multiple steps after the main enzymatic reactions and suffer interferences of various biological intermediates. A microtiter plate test based on D-amino oxidase (DAAOx) and horseradish peroxidase (HRP) O-phenylenediamine (OPD) chromogenic reaction was particularly developed to D-alanine assays and further improved by fluorogenic horseradish peroxidase coupled reactions (Gutheil et al., 2000). Although, in principle, the test is supposed to be flexible for other amino acids, no attempts were made to confirm it. Additionally, this technology still requires the use of more expensive black-sided microtiter plates and a fluorescence-based microtiter plate reader.

We describe here a simple and reliable microtiter plate test for the determination of most D-amino acids that can be readily adapted for the analysis of specific amino acid racemase activities. This test took into account that D-amino oxidase oxidizes several (but not all) D- amino acids and generate stoechiometric amounts of hydrogen peroxide. This very important parameter has a strong impact on the choice of the substrate that should be used to develop the reaction since it may affect the assay sensitivity. Here, the quantitation of D- amino acids by simple colorimetry was further possible by the inclusion of the highly sensitive HRP and a chromoreagent such as OPD. Furthermore, as an example of additional applications, the present DAAOx test was adapted for the medium/high throughput screening of Proline racemase specific inhibitors.

Materials and Methods

Reagents and buffers

Solution A consisting of 0.2 M sodium acetate/0.016 M sodium pyrophosphate (Pop) buffer, pH 8.3 is used to prepare the enzyme stock solutions preserved in 50% glycerol, namely: 50 U/ml D-amino acid oxidase from pig kidney (DAAOx, Sigma) and 5000 U/ml Horse Radish Peroxydase (HRP, Sigma). Stock solutions of L- amino acids (1M), D-amino acids (1M) and Pyrrole-2-carboxylic acid (10 mM) (PYC, Sigma) are also prepared in solution A. 10^{-1} M FAD (Sigma) stock was prepared in water. Solution B consists in adding to solution A the following reagents at a final concentration in the assay of 1 U/ml for DAAOx, 0.5 mg/ml orthophenylenediamine (OPD, Sigma), 37.5 U/ml HRP and FAD ($1.5x10^{-5}$ M FAD). For racemization assays, solution A consists of 0.2 M Sodium acetate pH 6.0 buffer.

D- amino acid test (DAAox test) : The test is run in 96 wells microplates (Nunc, Denmark). In one step reaction, 50µl of each experimental or standard samples diluted in solution A are loaded per well previously containing 50µl of solution B. Plates are incubated for 1 hour at 37°C in the dark. The reaction is stopped by addition of 50µl of 3 N HCl to the wells. OD 490nm - 650nm is obtained with a microtiter plate reader (Molecular Devices), and compared to those obtained with blank control wells. Limit of detection (LOD) was defined as the lowest concentration of a given D-amino acid detectable or quantifiable in a sample. LOD was determined as the mean value of the negative (blank) controls (containing no D-amino acids) plus three standard deviation (SD) of the means. Limit of quantification (LOQ), or lower detected concentration of D- amino acids to be considered for a high degree of confidence in quantitative tests, was defined as the mean value of the blank controls plus 10 SD, as described (Armbruster et al., 1994). Sixteen replicate sample series containing 1.25 mM to 0.02 mM of D-proline were assayed by the DAAOx test in parallel with a series of blank (negative) samples to generate an analytical calibration curve. The mean OD values and the SD obtained for the different concentrations of D-proline and for the blank samples were calculated. Distribution of experimental values were used to calculate a regression curve : $< y = Ax + b >$, where "y" = OD signal, "A" corresponds to the slope of the line, "b" the calculated Blank and "x" = the unknown concentration of D- proline (mM). Coefficients of Variance (CV) were calculated from the cumulative mean and SD of the means of experimental samples, using the following formula $Cv = \sigma / \mu$, were σ is the SD and μ is the mean value.

Standards

Serial dilution of D- amino acids is used as standards. L-amino acids may react at very low rate with DAAOx. For instance, DAAOx reacts with L-Proline 300 times slower than D-Proline. Thus, when the test is meant to assess the concentration of D- amino acids generated by racemization, standards should consist of serial dilution of equimolar mixture of specific D- and L-amino acids ranging from 0 to 20 mM (10 mM final) to take into account any interference of possible reactivity with DAAOx of L- form of amino acids present in the samples.

Biological samples

Post mortem extracts of human pre-frontal cortex from two 74-83 year old normal subjects or from three 75-86 year old Alzheimer Braak VI patients, prepared in solution A, were kindly provided by Dr. P. Lafaye, Department of Immunology, Institut Pasteur. Supernatants were obtained after centrifugation of the samples for 15 mn at 15000 g at room temperature. All supernatants were adjusted to 1mg/ml of protein and 90 µg were used in the DAAOx test. The samples were analyzed individually.

Preparation of r*Tc*PRAC

Recombinant *Trypanosoma cruzi* Proline racemases (r*Tc*PRAC) was produced in E. Coli BL21 (DE3) (Invitrogen) and purified with immobilized metal affinity chromatography on nickel columns as previously described (Reina-San-Martin et al., 2000).

Racemization assays with L-Proline

Optimum Proline racemization conditions for recombinant *Trypanosoma cruzi* proline racemase (*Tc*PRAC) were determined using 10-20 mM L-Proline in 0.2 M NaOAc, as a function of pH, as described (Reina-San-Martin et al., 2000). Briefly, classical assays of Proline racemization induced by *Tc*PRAC were therefore performed in tubes in a final volume of 1.5 ml and optical rotations measured in a polarimeter 241MC (Perkin Elmer). Parallel assays of Proline racemization were transposed into microtiter plates in a final volume of 100 µl, as follows: serial dilutions of L- Proline ranging from 40 mM to 2.5 mM were prepared in 0.2 M sodium acetate pH 6.0 containing 0.25 mM r*Tc*PRAC and loaded into microtiter plate wells in the presence or in the absence of 0 - 20 µM pyrrole-2 carboxylic acid (PYC), the specific inhibitor of Proline racemases. Seventy eight samples of N- and O- heterocyclic synthetic organic compound derivatives of organic acids were obtained from ChemDiv Inc. (San Diego, CA, USA) were tested for their ability to inhibit proline racemization. Specific control wells were prepared omitting the *Tc*PRAC and/or the PYC. Microtiter plates were incubated for 30 minutes at 37°C. Enzyme activity was then blocked by heating the plate for 15 seconds in a

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microwave oven at 900 watts after shifting the pH of wells to 8.3 by addition of 6.8µl of 0.235 M sodium Pyrophosphate. 50µl of each well were used to test the presence of D-Proline using the DAAOx test described here above.

Results

Principles of the DAAOx –test to quantify D-amino acids

Given the high complexity of available methods to detect D-amino acids in biological samples and as an alternative to individual sample analysis by polarimetry, we have further developed a simple and reproducible one-step enzymatic test in microtiter plates to quantify most D- amino acids. The test relies on D- amino acid oxidase (DAAOx) and its ability to specifically oxidize several (but not all) D- amino acids resulting in stoechiometric amounts of hydrogen peroxide in the presence of FAD and oxygen. For instance, as can be seen in (Figure 1A), the reaction of D-amino acid oxidation by DAAOx reduces FAD, produces an α-ketoacid and releases ammonia. In the case of D-Proline (Figure 1B), FAD is reduced but the pyrrolidine cycle prevents the formation of α-ketoacid and no release of ammonia occurs.

In the presence of oxygen, $FADH_2$ is oxidized and generates hydrogen peroxide. In order to obtain a colorimetric reaction that can be used to quantify any D-amino acid concentration in a sample fluid, an additional reaction is required where precise amounts of hydrogen peroxide can be readily deduced by the addition of HRP and OPD. Thus, as shown in (Figure 1B) for D-Proline, the reaction of H_2O_2 with HRP/OPD induces the generation of (6 amino phenyl)-diazene, a colored product that absorbs light at a wavelength of 490 nm. Moreover, the optical density at 490nm is proportional to the D- amino acid amount present in a sample. In the present case for instance, two molecules of D-Proline yield two molecules of hydrogen peroxide that under HRP/OPD reaction enables the formation of one molecule of colored detectable product.

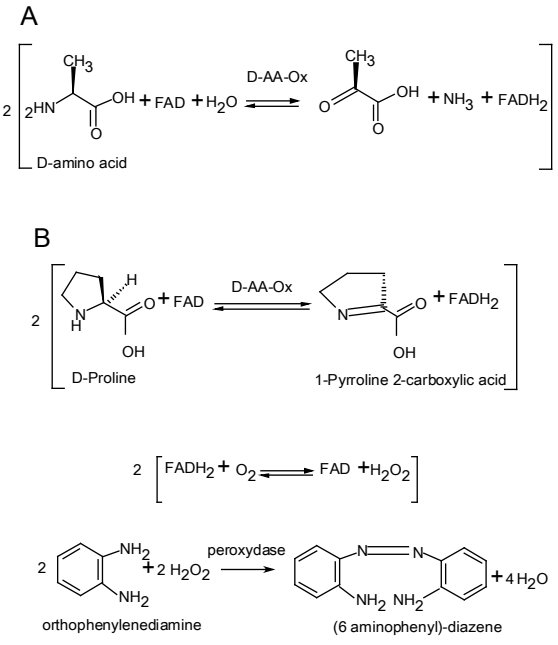

Figure 1: ***Principles of the DAAOx test.*** **A.** General scheme of amino acid oxidation. **B**. Particular case of D-Proline oxidation.

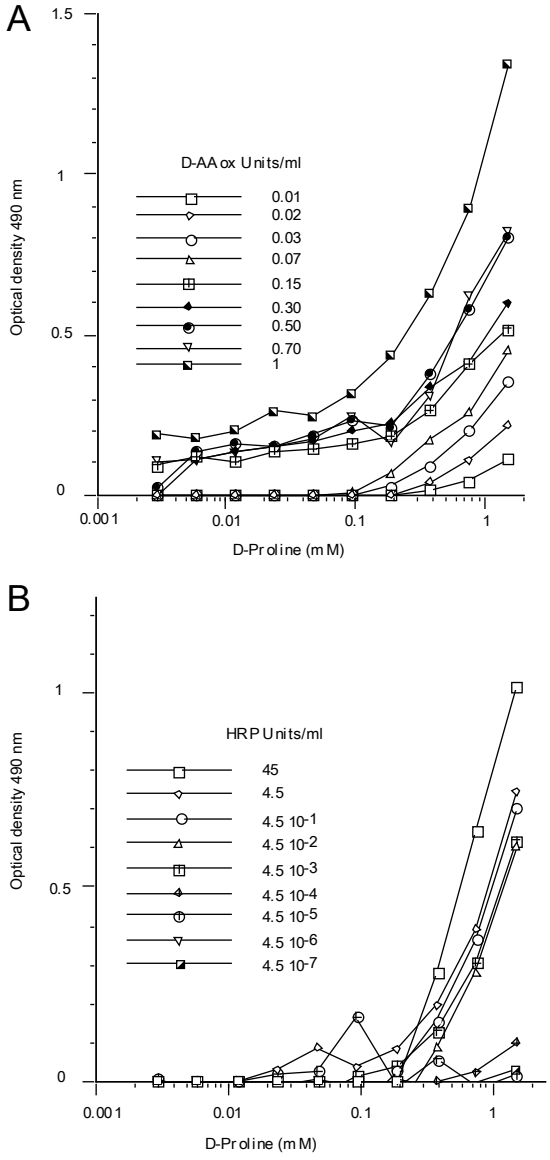

Figure 2: ***Determination of optimal D-AAox and HRP concentrations to the detection of D-amino acids in a DAAOx microtiter plate test.*** **A.** The assays were carried out using increasing amounts of D-Proline (mM) as a substrate and a fixed concentration of 37.5 Units/ml of HRP. Optical densities were obtained at 650/490 nm using a microtiter plate reader and correspond to increasing amounts of H_2O_2 revealed by OPD-HRP chromogenic reactions. Each point is the average of duplicate values from one single experiment that was repeated at least 3 times. **B.** The assays were carried out using a fixed concentration of DAAOx (0.9 Units /ml) and variable amounts of D-Proline (mM). Different concentrations of HRP were used to evaluate the appropriate amount required to detect D- Proline based on the release of H_2O_2. 490nm – 690nm optical densities of OPD-HRP chromogenic reactions were obtained using a microtiter plate reader. Each point is the average of duplicate values. The assays are representative of two experiments.

D-amino acid detection by the DAAOx in microtiter plates

In a first set of experiments we assessed the suitable concentration of DAAOx for D-Amino acid determination. Using serial dilutions of D-Proline as a substrate, reactions were set up using appropriate pH buffer conditions and concentrations of the DAAOx ranging from 0.01 to 1 unit/ml, while keeping constant the HRP concentration

(37.5 Units/ml). (Figure 2A) shows that the optical densities obtained from increasing amounts of formed H_2O_2 are proportional to the concentration of DAAOx used in the assay. However, as compared to the known amounts of substrate used in the reaction, compatible amounts of D-Proline were detected when using 1 unit/ml DAAOx. Later, while keeping constant the DAAOx concentration at 1 unit/ml, we have assayed the ability of different amounts of HRP (between 4.5 x 10^{-7} and 45 Units/ml) to form hydrogen peroxide originating from oxidized D-Proline. Data presented in (Figure 2B) shows that the threshold for color reaction is 4. 5 x 10^{-3} Unit/ml HRP. As for the DAAOx, the levels of hydrogen peroxide obtained are proportional to the concentration of HRP used in the reaction. Therefore, the optimum concentration of HRP was defined as 45 Units/ml.

Affinity of the test to different D- amino acids

It has been reported that some D-amino acids are not oxidized by D-AAOx (D'Aniello et al., 1993; Murachi and Tashiro, 1958). In order to assess the substrate specificity of the D-AAox/HRP test to react with different D- amino acids we performed the assay using serial individual dilutions (90µM to 1.25 mM) of different D- amino acids. As can be seen in (Figure 3), the enzyme presents a marked preference for hydrophobic aliphatic amino acids such as Proline, Isoleucine, Alanine, Leucine, Tyrosine, Methionine and Phenylalanine. However, anionic (Aspartic acid and Glutamic acid) or cationic (Histidine, Lysine and to a lesser extent Arginine) charged amino acids, as well as the polar non charged Threonin are poor substrates for DAAOx. Other amino acids, either polar (Serine) or hydrophobic (Valine) or both (Tryptophan), react with the enzyme at an intermediary level between the two former groups. Therefore, three groups can be distinguished in relation to their reaction rate with the enzyme.

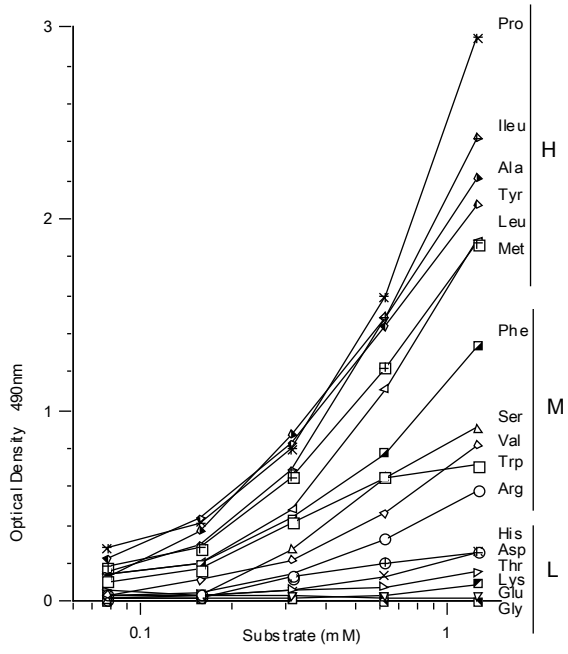

Figure 3: ***Affinity of DAAOx for various D-amino acids.*** The DAAOx test was carried out using the D- form of 16 Amino acids and Glycine as substrates. Serial dilutions of each amino acid starting at 1.25 mM were loaded in the microtiter plates. Appropriate controls using L-forms of the amino acids were used as negative reaction controls (not shown). 490nm – 690nm optical densities corresponding to the amounts of H_20_2 produced from oxidation of different amounts of the substrates are presented in Y axis. High, medium and low rate DAAOx-reactive amino acid groups are represented respectively by H, M and L.

Accordingly, a "high rate" group of D-amino acids comprises Proline, Isoleucine, Alanine, Methionine, Leucine, Tyrosine and Phenylalanine that are very good substrates to the catalytic pocket of DAAOx. A "medium rate" group, encompasses Serine, Valine, Tryptophan and Arginine. The "very low rate" group includes D-amino acids such as Histidine, Aspartic acid, Lysine, Threonine, Glutamic acid that show poor or no reactivity with DAAOx/HRP, corroborating previous data (D'Aniello et al., 1993). It is worth noting that Glycine, that lacks chirality, is not oxidized by DAAOx and cannot be determined by the present test. Appropriate controls using high and low concentrations of L-amino acids corresponding to the 3 reactive groups were tested and none (i.e. Proline, Tyrosine, Valine, Threonine, Tryptophan, Lysine and Glutamic acid) gave any detectable reaction under the test conditions. It was previously shown that the total D-amino acids' contents (mostly, Aspartate and Alanine) were significantly higher in the cerebrospinal fluid and frontal cortex of Alzheimer's diseased patients (AD) (D'Aniello et al., 1992; Fisher et al., 1998). Indeed, considering that Aspartic acid shows poor or no reactivity with DAAOx, the present method confirmed that free D-amino acid levels are considerably higher in the human frontal cortex extracted from AD brains (490 ± 5.0 nmol/g) than those of control brains (305 ± 10.0 nmol/g).

Application of the test to study amino acid racemases and their potential inhibitors

With the goal of developing an alternative method to polarimetry to measure the enzymatic activity of a particular amino acid racemase in microtiter plates, we verified if the test could be used to determine the amount of D-amino acids formed in reactions where the L-counterpart is incubated with such an enzyme in the presence or in the absence of a known specific inhibitor. We then adapted the DAAOx test to reproduce the ability of pyrrole carboxylic acid (PYC) to specifically inhibit *Tc*PRAC, property primarily described for the proline racemase of *Clostridium sticklandii* (Keenan and Alworth, 1974). At first, in order to take into consideration all possible minor interferences caused by L- amino acids that are also capable of reacting with DAAOx but with a 300 times slower rate than the D- forms, the standard of the test was prepared with equimolar amounts of L- and D- Proline (see methods). The technique was set up integrating the results here above showing that DAAOx presents high affinity to Proline, the substrate of *Tc*PRAC. Additionally, considering that D-Proline is a substrate for both *Tc*PRAC and DAAOx, it could be hypothesized that an inhibitor of the catalytic pocket of *Tc*PRAC, which is a structural analog of Proline, could inhibit DAAOx activity as well and, therefore, interfere with the assessment of new putative inhibitors of such a racemase. To test this hypothesis we determined the impact of a known Proline racemase competitive inhibitor on DAAOx activity. We thus, analyzed the abilities of the test to determine serial dilutions of equimolar mixtures of D/L-Proline in the presence of known dilutions of PYC, a structural analog of the planar intermediary form between L- and D-Proline, and this in the absence of *Tc*PRAC. As can be observed in (Figure 4A), close to 100 % inhibition of D- Proline oxidation is observed when the test was set up with increasing amounts of PYC and up to 5 mM D/L proline, indicating that the inhibitor is able to interfere with DAAOx activity. Nevertheless, minor interference is observed whenever reactions were set up with higher concentrations of substrate (i.e. 10-20 mM D/L Proline), probably due to a competition between the substrate and the inhibitor for the DAAOx active site. However, no DAAOx interference by PYC is observed at concentrations of the inhibitor below 20µM regardless of the substrate concentration. These data

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suggest that the optimal conditions of the present test to detect D- amino acids generated by a given amino acid racemase should be determined individually and according to possible interferences

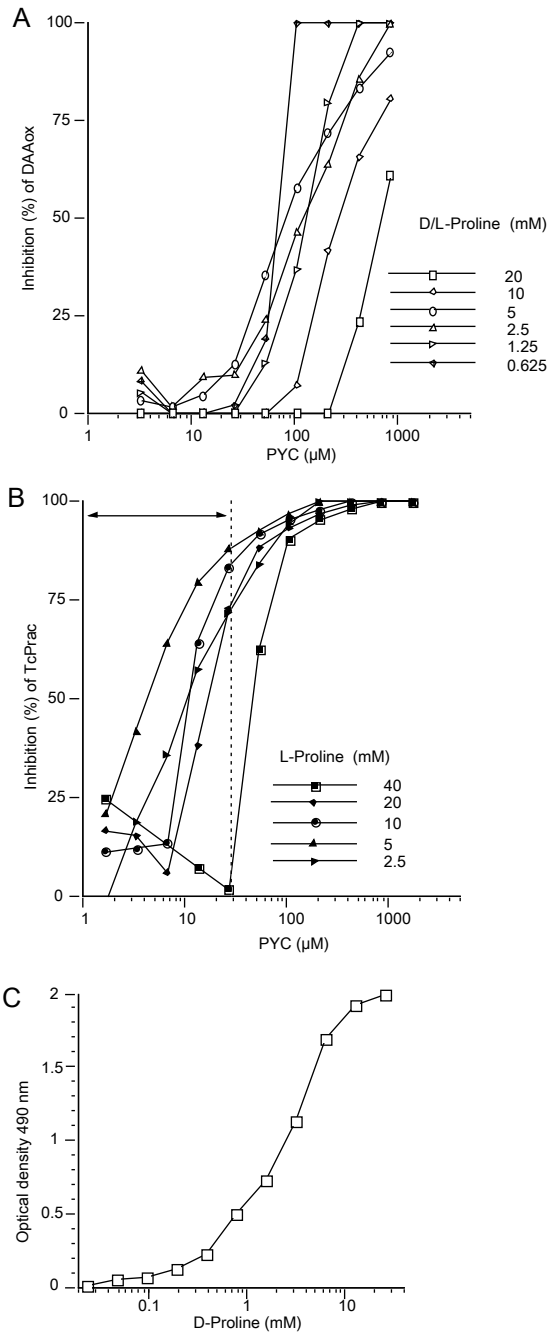

Figure 4: ***Inhibition of TcPRAC enzymatic activity by the specific inhibitor, pyrrole-2 carboxylic acid (PYC), evaluated with the DAAOx test.*** **A.** D- Proline detection by the test is hindered by the inhibition of the DAAOx activity observed with increasing amounts of PYC in reactions were Proline racemase was omitted, **B.** The optimal window for specific analysis of *Tc*PRAC inhibition without interference on DAAOx enzymatic activity is double arrowed and limited in the figure by the dotted line, **C.** A standard curve is made with various concentrations of the required substrate (mM D- Proline here). The test was carried out using fixed concentrations of DAAOx (0.9 Units /ml) and of HRP (37.5 Units/ml). Optical densities corresponding to the amounts of H_2O_2 produced from DAA oxidation of different amounts of D-Proline is presented in Y axis. Using a semi-logarithmic representation, the picture depicts the linear part of the curve obtained between 8 mM and 0.3 mM D-Proline.

	Racemization (%)		D-Proline (mM)	
L-Proline (mM)	Polarimetry	DAAOx	Polarimetry	DAAOx
40	44	42	8.8	8.4
20	59	50	5.9	5.0
10	64	64	3.2	3.2
5	50	60	1.2	1.5
2.5	100	90	1.2	1.1

[a]Serial dilutions of L-Proline (mM) were incubated for 30 minutes at 37°C in test tubes with or without 0.25 µM *Tc*PRAC in 0.2 M sodium acetate pH 6. Reactions were stopped by heating at 65° C for 10 minutes. Fifty microlitres of each sample were tested for D-Proline contents by the DAAOx test. The remaining reaction volume (1.1 ml) was analyzed for D-Proline formation by polarimetry at 365 nm.

Table 1: Comparison of polarimetry and DAAOx methods to determine variable amounts of D-Proline resulting from *Tc*PRAC racemization reactions[a].

with DAAOx of a known specific racemase inhibitor. A second set of experiments was then delineated to verify the window of specific activity of PYC toward *Tc*PRAC, i.e. where no blockage of DAAOx activity is observed. Reactions were then performed using known amounts of L-Proline and of *Tc*PRAC in the presence of serial dilutions of PYC. As shown in (Figure 4B) and related to the amounts of D-Proline determined by the test, about 50% of specific inhibition of *Tc*PRAC racemization is observed when using 4 – 30µM PYC and according to the initial concentration of L-Proline (i.e. 2.5 – 20 mM). Therefore, the test described in this context is suitable for the assessment of *Tc*PRAC putative inhibitors with an IC50 ranging from 4 – 30µM.

Comparison of D-Proline determination by the D-AAox test and by polarimetry

In order to compare the sensitivities of the microtiter plate and the polarimetric test in determining D-Proline, and to validate the DAAOx test to screen proline racemase inhibitors, we have used both methods to determine D- Proline contents in samples from racemization assays using *Tc*PRAC and L- Proline as substrate (Reina-San-Martin et al., 2000). *Tc*PRAC racemization reactions were set up using variable concentrations of L-Proline substrate and the resulting levels of D- Proline were then evaluated by polarimetry and by DAAOx test. Table 1 shows a good agreement between the two methods in that they reveal comparable amounts of D- Proline. In addition, lower concentrations of D- amino acids may be determined by the present test as exemplified by the highly sensitive determination of D- Proline as compared to polarimetry. Thus, while as low as 300 µM of D- Proline may be precisely quantified by the DAAOx method, underestimated values of D-Proline may be obtained at higher concentrations of substrate i.e. > 10 mM, which correspond to the racemization of 10 mM L-Proline, as asserted by the semi-log plot shown in (Figure 4C). This data suggests that less than 20 mM substrate should be used for the assay.

To calculate the limit of detection (LOD) of the test we tested increasingly more dilute concentrations of D- proline to better consider the distribution of the analytical signal (OD) obtained for repeated measurements of blank samples and the value distribution from samples containing lower proline concentrations. The mean OD value and SD of a series of blank samples collected from microtiter plate tests of 16 replicates of serial dilution of D- proline (from 2.5 mM to 0.02 mM) were calculated (0.1546 ± 0.0093). LOD (mean + 3 SD) and LOQ (mean + 10 SD) values were then converted to D-proline concentrations by subtracting the blank calculated signal and dividing by the slope of the analytical calibration curve (y = 1.3653x + 0.1324, see Methods) and correspond respectively to 37µM and 84µM. The CV of the raw data obtained from these calculations (6%) indicates that the values represent a high confidence limit for the blank and that any value higher than the LOD is likely due to the presence of

quantifiable amounts of D-proline, statistically distinguishable from "zero".

Simplified description of a high throughput D- amino acid oxidase method adapted for the evaluation of proline racemase activity

Based on the results shown here, we have further developed a baseline method for microtiter plates to detect and quantify most D-amino acids resulting from the activity of an amino acid racemase. Furthermore, using the above determined PYC concentration range (up to 20µM) that prevents interferences with DAAOx, the methodology was made applicable to assess the activity of proline racemases and to identify potential enzyme inhibitors. Briefly, racemization assays are set up in microtiter plates using 10 mM *Tc*PRAC, 10 mM L-proline in a final volume of 100µl of 0.2 M sodium acetate pH6 in the presence or in the absence of PYC (5-10µM), or of synthetic compounds identified from virtual screening of chemical libraries with a *Tc*PRAC-specific PYC-based pharmacophore (not shown). After 30 minutes incubation at 37°C the enzyme was inactivated by heating (see methods). Fifty microliters of samples, blank wells containing only L-proline or equimolar amounts of L-/D-proline standards were transferred to a new flat-bottomed microtiter plate and diluted with 50µl of pH 8.3 buffered solution containing 1.5 10^{-5} M FAD, 0.9 Unit/ml DAAOx, 45 Units/ml HRP and 0.5 mg/ml OPD and incubated for one hour at 37°C in the dark. Reactions are stopped by the addition of 50µl of 0.3 N HCl. Chromogenic reactions are then read at 490 nm - 0.D. 650 nm using a microtiter plate reader. (Figure 5A) depicts an example of a microtiter plate template where seventy eight synthetic compounds were analyzed for their ability to inhibit the racemase activity of *Tc*PRAC in comparison to wells containing or not IC50 of PYC (10µM). As can be observed in the template, wells from one plate column consist of serial dilutions of 0-10 mM of L-/D- proline standards (STD). Out of 78 unknown (Unk) molecules distributed in the microtiter plate, microwells C7 and D7 contain duplicate racemization reactions performed in the presence of PYC (10µM). Optical densities obtained at 490-650 nm are shown in (Figure 5B). Considering that the *Tc*PRAC preparation used in the test had previously exhibited 98% activity by polarimetry, and that the reaction was diluted 1:2 to perform the analysis of D-proline by the DAAOx test, only 2.5 mM D-proline should be expected in wells without inhibitor. This was indeed the case for E1 and F1 wells (w/o PYC), given that the obtained analytical signals correspond to the OD observed for 2.5 mM in the STD curve (well C12). These results differ with the 50% inhibition of the proline racemization observed in wells containing the specific PYC inhibitor (G1, H1, C7 and D7), where only 1.25 mM D-proline was measured. No other compounds used in the test were shown to inhibit proline racemase, since the OD values obtained for the different reactions are compatible with the 2.5 mM D-proline signal observed in the well C12 of the STD curve. This methodology showed a good reproducibility for several hundred of molecules tested to date and represents a reliable tool to screen a large number of compounds.

Discussion

In the present work we describe a one-step microtiter plate method to quantify most D-amino acids based on the enzyme D-amino acid oxidase. The enzyme catalyses the oxidation of D- amino acids leading to the generation of stoechiometric amounts of hydrogen peroxide that can be further quantified by HRP-OPD reaction. More than a mean of testing the presence of D-amino acids in samples or tissues of individuals afflicted or not with various pathologies, the simplicity of the present test is consistent with its use to evaluate amino acid racemization reactions or as a medium/high throughput methodology to screen, for instance, amino acid racemase inhibitors.

The interest in the identification of D- amino acids has increased in the last decade since certain pathological and physiopathological processes have been shown to be related to modifications of the free L- or D- amino acids pool in some organs and fluids of mammals, including humans (Fisher, 2007). In addition, the discovery of naturally occurring free or liganded D-amino acids in biological fluids, tissues and organisms (Coatnoan et al., 2009; Konno et al., 2007) has also contributed to the development of several analytical methods

A

→ *Tc*PRAC ----------→

												STD L/D-Pro (mM)
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	**Blk**	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	10
B	**Blk**	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	5
C	**Blk**	Unk	Unk	Unk	Unk	Unk	**PYC**	Unk	Unk	Unk	Unk	2.5
D	**Blk**	Unk	Unk	Unk	Unk	Unk	**PYC**	Unk	Unk	Unk	Unk	**1.25**
E	**(-) PYC**	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	0.625
F	**(-) PYC**	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	0.312
G	**(+) PYC**	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	0.156
H	**(+) PYC**	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	Unk	0.078

B

Optical densities at 490-650 nm

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	0.006	1.242	1.242	1.276	1.216	1.290	1.261	1.256	1.326	1.254	1.360	3.250
B	0.002	1.360	1.255	1.352	1.344	1.204	1.223	1.352	1.355	1.351	1.242	2.560
C	0.005	1.313	1.310	1.236	1.256	1.366	**0.520**	1.236	1.365	1.305	1.333	1.364
D	0.001	1.362	1.351	1.365	1.262	1.332	**0.625**	1.211	1.206	1.330	1.204	**0.554**
E	1.300	1.350	1.232	1.223	1.251	1.266	1.266	1.245	1.313	1.262	1.350	0.256
F	1.253	1.205	1.350	1.306	1.205	1.204	1.360	1.263	1.366	1.342	1.240	0.104
G	**0.554**	1.302	1.204	1.360	1.266	1.342	1.264	1.365	1.322	1.365	1.365	0.062
H	**0.646**	1.325	1.263	1.355	1.355	1.266	1.342	1.302	1.355	1.204	1.341	0.023

Figure 5: ***High throughput application of the DAAOx test for the identification of TcPRAC inhibitors.*** **A.** Baseline template for a microtiter plate : Blank wells (Blk), Pyrrole carboxylic acid (PYC), Unknowns (Unk), Equimolar final concentration of L/D proline (mM) standard (STD). **B.** Optical densities obtained at 490-650 nm and corresponding to reactions performed in the microtiter plate presented in Figure 5A, in the presence (+) or in the absence (-) of known and unknown inhibitors of *Tc*PRAC.

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applied to quantify their levels in physiological and pathological conditions, as well as in the fermentation of food and consequently in biotechnology.

The presently developed microtiter plate test based on the D-amino oxidase and peroxidase enzymes offers several advantages over the previous available methodologies. Firstly, it provides a quick and low cost method that gives a global and reliable quantification of D-amino acids in a sample. Additionally, several samples can be processed simultaneously in a microtiter plate making the DAAOx test a method of choice for screening multiple samples that potentially contain D- amino acids. Secondly, DAAOx method facilitates the determination of D- amino acids without losses or artifacts encountered due to the processing of the sample on HPLC or chiral gas chromatography. The method has an additional advantage over that previously described by Nagata to determine by DAAOx the increase of D- amino acids in the serum of patients with renal diseases or senescence. Nagata's method was only suitable for D- amino acids that produce α-keto acids (Nagata et al., 1985; Nagata et al., 1992), which is not the case, for instance, of D-Proline. Moreover, Nagata's procedure implies two distinct and sequential reactions, which cannot be run simultaneously, thus lengthening the test as compared to the present method. Alternative to the detection of α-keto acids emanating from the oxidation of certain D- amino acids, the present test devised the quantification of another reaction product, the hydrogen peroxide, by the use of peroxidase and the O-phenylenediamine reducing agent. Other reducing agents can be envisaged to enhance the sensitivity of the test to some D- amino acids such as 3,3', 5,5'-tetramethyl-benzidine (Madersbacher and Berger, 2000).

While the current DAAOx test is very sensitive to determine the total amount of D- amino acids in a sample, it presents some limitations, such as the fact that some commercialized D- amino oxidase present a relative selectivity to certain D- amino acids. Thus, we have shown here that DAAOx from pig kidney displays a marked substrate preference for hydrophobic aliphatic amino acids such as Proline, Isoleucine, Alanine, Leucine, Tyrosine, Methionine and Phenylalanine and poor reactivity with charged amino acids (Aspartic acid, Glutamic acid, Lysine, and, to a lesser extent, Histidine) and also with the polar Threonine. Therefore, in contrast to D- Alanine and D- Serine, amino acids such as D- Aspartate and D- Glutamate which are equally involved in food/beverages quality controls cannot be quantified by DAAOx methods. However, this limitation can be circumvented by using microbial D-amino oxidases which have a broader range of substrates or, for instance, by specific D-aspartate oxidase (Dixon and Kenworthy, 1967; Gabler et al., 2000; Pollegioni et al., 2008; Yagi et al., 1969).

One of our specific goals was to improve the methods to analyze amino acid racemization *in vitro*. For instance, as mentioned previously, very complex methodologies were available to analyze amino acid racemase activities and search for putative specific inhibitors. However, the samples are analyzed individually and mainly use polarimetry, a technique that is time-consuming and thus inadequate to test several hundreds of molecules originated by virtual screening of chemical libraries. The DAAOx method described here revealed to be a suitable medium/high throughput microtiter plate technique to rapidly investigate hundreds of potential inhibitors of, for instance, a parasite proline racemase (*Tc*PRAC) that was validated as a therapeutic target against American trypanosomiasis (Chamond et al., 2005). Thus, we have shown that the resulting amounts of D-Proline determined by the test in samples that were submitted to L-Proline racemization by *Tc*PRAC were comparable to those obtained by polarimetry. Furthermore, the method was effective to unveil the inhibition of *Tc*PRAC by its competitive inhibitor (PYC), in a reaction range that does not affect the DAAOx activity. Therefore, we suggest that beyond the rapid determination of D-amino acid in fluids, either in agro-alimentary controls or in biological research samples the present medium/high throughput test may be useful to the identification of amino acid racemase inhibitors in general.

Conclusion

We have designed a single step medium/high throughput enzymatic method in microtiter plates to detect and quantify most D-amino acids in biological fluids, samples and lysates. Using a previously determined inhibitor concentration range that prevents interferences with DAAOx, the test is applicable to assess inhibitors of amino acid racemases and consequently of D-amino acid formation.

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