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Highly Sensitive Determination of DAO Activity by Oxidation of a Luminescence Reagent

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Abstract A highly sensitive method for measuring the activity of the enzyme diamine oxidase (DAO) independent of the type of substrate is described. The principle of the assay is to determine the amount hydrogen peroxide generated as a reaction product during oxidation of diamines by DAO. PSatto™, a highly sensitive luminescence reagent, was used to generate a signal depending on the hydrogen peroxide concentration based on the action of horseradish peroxidase. DAO is specifically captured from a sample by an antibody immobilized to microwell plates, and the substrate is added to the bound enzyme. Various diamines were used as substrates; the peroxide produced is directly proportional to the amount of DAO bound to the specific antibodies. With this very sensitive method, it is possible to detect pmol amounts of generated hydrogen peroxide in plasma matrix corresponding to the biological activity of DAO.

Keywords Diamine oxidase · Luminescence · Putrescine · Histamine · Antibody · Purification

Introduction

Amine oxidases play an important role in the regulation of diamine levels in animals and plants. Degradation of biogenic amines is of eminent physiological importance; hence, amine oxidases represent a relatively conserved group of enzymes. Diamine oxidase (DAO; EC 1.4.3.6.) is one of the most prominent member of this group in mammals because it degrades histamine and other biogenic amines like putrescine [1], which have various regulative and modulating functions.

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DAO belongs to the class of copper-containing amine oxidases, which catalyze the oxidative deamination of primary amines by dioxygen to form aldehydes, ammonia, and hydrogen peroxide [2].

In many mammals, this enzyme is mainly responsible for the degradation of histamine ingested with food [3, 4]. The structure, expression, activity, and function of porcine DAO were analyzed in several studies [5–8].

Native DAO from porcine kidney is a homodimeric glycoprotein with subunits of a relative molecular mass of approximately 85 kDa [9, 10] linked by disulfide bonds [11], it contains the active site cofactor topaquinone, formed posttranslationally by modification of a conserved tyrosine residue and has a carbohydrate content of 11% [12].

As the biochemical functions of the intestine and kidney in omnivores are quite similar, many studies on DAO were performed using the porcine system [13, 14].The major sites of expression are the placenta, kidney, and intestine [15].

One fundamental function of DAO in the small intestine is to prevent the uptake of ingested histamine and other biogenic amines from the intestine into circulation. This is an important step for the control of intestinal and blood concentrations of histamine. DAO as a first barrier against orally ingested diamines is of vital importance for the effective degradation of this class of substances.

Experimental inhibition of DAO and food challenged with commercially available cheese and wine in pigs induced anaphylactic reactions in each animal and death in 20% of the pigs [16, 17].

Normal human plasma levels range from 15 to 50 U/ml. Human plasma DAO is elevated up to 500-fold during pregnancy [18] This elevated activity is caused by the production of DAO in the placenta inhibiting unwanted contractions of the uterus initiated by high histamine concentrations, e.g., after consumption of histamine-rich food. During pregnancy, the course of migraine is effected positively. Also, food-associated headache and allergic symptoms are normally relieved in pregnancy with recurrence some weeks after delivery.

Remissions of symptoms concerted with the decrease of DAO activity indicate that histamine is an important factor for the occurrence of these symptoms.

These findings confirm the importance of DAO activity in mammals. In the past, many assays were developed to quantify DAO activity but most of them are not sensitive enough for detection of physiological concentrations, e.g., in plasma samples [19–22]. The most common method for the determination of DAO activity in serum and plasma was first described by Tufvesson and Tryding [23]. This assay is based on the conversion of radioactively labeled putrescine and remained the only relevant one for years. Even the first commercially available assay was based on this method [24]. Many laboratories try to avoid radioactivity because of legal restrictions and high costs for storage of radioactive waste.

Therefore, there is a need for a nonradioactive, highly sensitive assay for the quantification of DAO activity in serum and plasma.

Reaction Scheme of Diamine Oxidase Catalyzed Reactions

As shown in Fig. 1, DAO catalyzes the oxidative deamination of primary amines by dioxygen to form aldehydes, ammonia, and hydrogen peroxide [25].

In the test system described in this study, the generated hydrogen peroxide acts as substrate for a very sensitive luminescent reagent. Using horseradish peroxidase as a converting enzyme, a luminescence signal directly proportional to DAO activity in the sample is generated. The test is easy to perform and even low activities of diamine oxidase can be quantified with this single step assay.

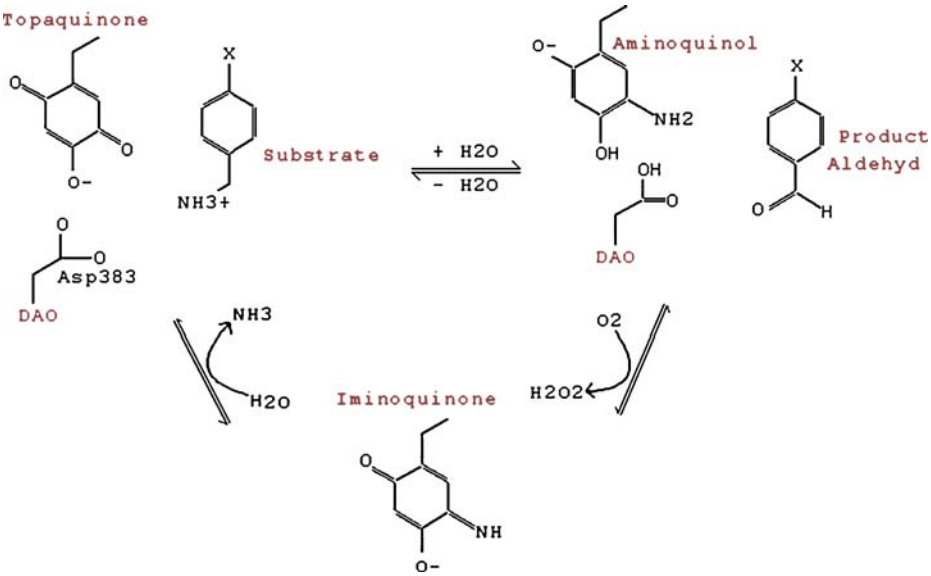


Fig. 1 Scheme of the reaction catalyzed by copper containing amine oxidases

Materials

Diamine oxidase from porcine kidney, horseradish peroxidase, hydrogen peroxide, putrescine, histamine, PMSF, caprylic acid, 4-nitrophenyl phosphate tablets, and all buffer substances were obtained from Sigma.

HiTrap affinity columns, phenyl sepharose, and Q-sepharose were from Amersham, cellufine sulfate was from Millipore, NuPAGE™ 4–12% Bis–Tris gel was from Invitrogen, and luminescence substrate PSatto™ solution B was obtained from Lumigen Inc USA.

NUNC Maxisorp high-binding microwell plates were purchased from VWR Austria.

Methods

Purification of Diamine Oxidase from Porcine Kidney

Porcine kidneys were mixed with an equal volume (*w*/*v*) of 20 mM phosphate buffer pH 7.5, including 1 mM PMSF in a blender and homogenized by an ultraturrax. The diamine oxidase was concentrated from the homogenate by fractionated ammonium sulfate precipitation. Of the activity in the homogenate, >70% could be found in the pellet of 72% ammonium sulfate. This pellet was redissolved in PBS for further purification. The majority of the proteins precipitated together with the enzyme could be separated from DAO by hydrophobic interaction chromatography (HIC) with phenyl sepharose. Fractions of 1 ml were collected, and activity was determined. HIC fractions with the highest activity of DAO were further purified on a QAE-column. DAO activity was quantified by radioextraction assay as described by Mayer et al. [24].

The last purification step was done on a cellulose sulfate column.

Purity of the enzyme was proven by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) the protein was used for immunization of chickens.

SDS-PAGE

Protein solution was mixed 1:2 (*v*/*v*) with 2x Laemmli buffer (0.5 M Tris–HCl; pH 6.8; 10% *w*/*v* SDS, 0.1% bromophenolblue, 20% glycerol, 5% β-mercaptoethanol). NuPAGE™ 4–12% Bis–Tris gel was put in a chamber with MES-buffer (50 mM MES, 10% SDS, 50 mM Tris, 0.8 mM EDTA). 10 μl of the sample was loaded onto the gel.

After electrophoresis, gel was stained with Coomassie blue.

Immunization of Laying Hens

Derco brown laying hens were purchased from Heindl (Vienna, Austria) and maintained on layer's mash with free access to water and feed under a daily light period of 16 h. For primary injection, the antigen was mixed with Freund's complete adjuvant, and the resulting emulsion was applied by intramuscular injection. All subsequent injections were carried out at intervals of 3 weeks each, this time using Freund's incomplete adjuvant. Eggs were collected from second boost injection and pooled per week and per hen. Antibodies were isolated from egg yolks. The concentration of specific antibodies in the yolks obtained was determined by enzyme linked immunosorbent assay (ELISA).

Purification of IgY

The egg yolks from the immunized hens were diluted with ninefold amount of deionized aqua. After adjusting the pH to 4.9, yolk proteins were precipitated with caprylic acid. Antibodies (IgY) do not precipitate under these conditions. Antibodies from the supernatant were further purified by ethanol precipitation. The pellets were finally suspended in PBS buffer and the antibody solution was kept at −20 °C until further use.

Protein-Coating on Microtiter Strips

Protein solution (yolk antibody or DAO) was diluted in carbonate buffer (20 mM Na–carbonate; 0.1% Na–Acid; pH 9.6) to 1 μg protein/ml. Microtiter wells were incubated with coating solution (200 μl per well) overnight at 4 °C, then the coating solution was poured off, and the well was incubated for 1 h at room temperature with 350 μl of blocking solution (0.1% BSA; 5% sucrose in carbonate buffer). Finally, the blocking solution was discarded and wells were dried and stored in an exsiccator.

Determination of Anti-DAO Titer of the Chicken Antibody

The IgY solution after ethanol precipitation was incubated on antigen-coated wells overnight. Wells were washed five times with WPL buffer (10 mM Tris; 0.01% Brij 35) followed by 1 h incubation at 37 °C with anti-IgY conjugate diluted in APF buffer (10 mM HEPES, 100 mM guanidine, 0.1% gelatine, 0.05% Brij 35, 0.17% thimerosal). After washing the wells again with WPL buffer, 100 μl of the substrate solution (4-nitrophenyl phosphate=1 mg/ml) was added per well.

The absorption at 405 nm was measured after about 10 min incubation in the dark.

Purification of DAO-Specific IgY by Affinity Chromatography

DAO was coupled on a HiTrap NHS-activated Sepharose™ High Performance column from Amersham as described in the instruction sheet. The IgY solution after ethanol precipitation

was diluted in 50 mM phosphate buffer pH 7.5 with 2% PEG before loading it onto the affinity column. Elution of specific antibodies was achieved with 200 mM glycine at pH 2.2. The eluted fractions were neutralized immediately with an equal volume of 1M Tris pH 8.

Results

Characterization of PSatto™ Solution B

Luminescence substrates are used for the detection of low concentrations of hydrogen peroxide. PSatto™ solution B proved as a very sensitive substance with low background signals compared to Luminol (Fig. 2).

The flash luminescent signal develops very fast within the first 2 s after the addition of PSatto™ solution B, is quite constant for 20 s, followed by a rapid decrease in 2 min (Fig. 3). The signal to noise ratio has an optimum of about 25 s after the addition of the substrate.

For maximum sensitivity, luminescence must be read immediately after the addition of the substrate. We used a PhL photoluminometer (Aureon Biosystems, Vienna, Austria) equipped with two Oyster Bay dispensers.

The detection limit of the system (120% signal/noise) was calculated to be 6 nM. This corresponds to 1.1 pmol of hydrogen peroxide per well.

This sensitivity is sufficient for the determination of DAO activity in serum or plasma samples.

Determination of DAO Activity in Buffer Matrix

PSatto™ shows high reactivity with most of the oxidants. To avoid any nonspecific signal, it is necessary to use a reaction matrix, which is essentially free of peroxides and other interfering substances. DAO was purified from porcine kidney by fractionated ammonium sulfate precipitation followed by three chromatographic steps, resulting in a purity of more than 90% (method described above; see Fig. 4), and was used for all following experiments.

DAO obtained from Sigma contained too many other proteins and substances, which affected the luminescence signal dramatically.

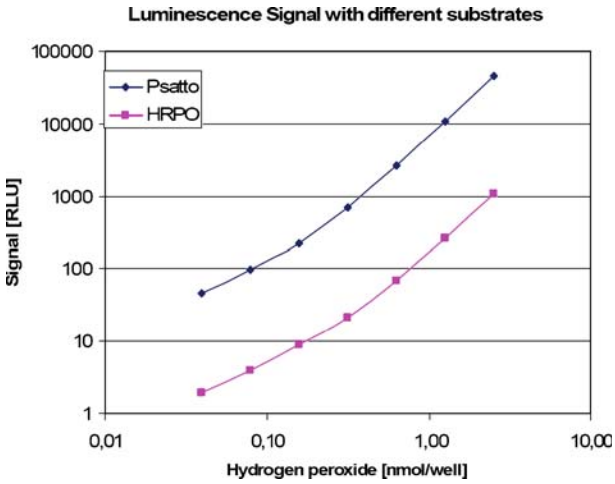


Fig. 2 Comparison of luminescent substrates

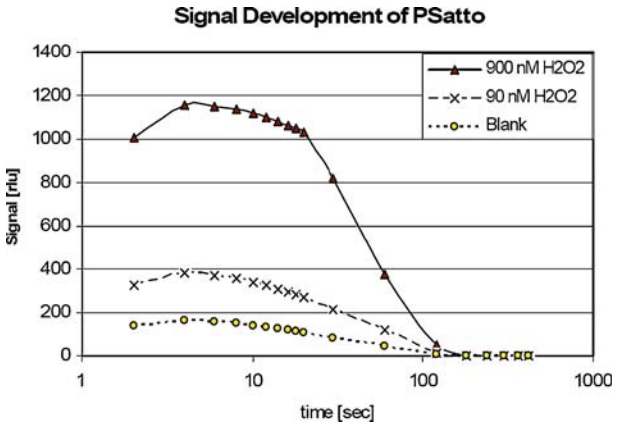


Fig. 3 Flash signal development

The stability of hydrogen peroxide in solution, which is influenced by various substances and temperature, has to be considered.

As described in many papers, diamine oxidase shows high conversion rates mainly for the aliphatic diamines putrescine and histamine [26–28].

We tested histamine and putrescine in different concentrations to determine substrate preferences and optimal substrate concentrations for our DAO preparation.

All reagents were diluted in 50 mM Tris pH 8.0. DAO was used in a serial dilution from 19 to 500 U/ml. 1 Unit in our system corresponds to 70 μU/ml as specified by Sigma (defined as oxidation of 1 μmol Putrescin/hour at pH 7,2 at 37°C). 50 μl of enzyme solution were incubated with 50 μl of varying substrate concentrations for 1 h at 37 °C. In additional, 20 μl horseradish peroxidase (HRPO) and 50 μl of PSatto™ were added to the reaction mixture. Luminescence signal was integrated for 10 s after a lag time of 5 s. DAO-specific luminescence signal depended on substrate concentration. DAO activity is inhibited at a substrate concentration higher than about 30 μmol/l, independent of the substrate. Furthermore, conversion of putrescine is about four times more efficient than histamine (see Fig. 5).

Incubation Conditions

Different incubation conditions were tested to improve assay sensitivity: 100 μl DAO solution in 50 mM Tris pH 8.0 was mixed with 50 μl putrescine (16 μM) and incubated at different temperatures for 30, 60 and 180 min. Subsequently, HRPO and PSatto™ were added with the dispenser. Finally, luminescence signal was integrated for 10 s after a delay time of 2 s.

Hydrogen peroxide is produced continuously during the action of the enzyme and accumulates in the reaction mixture. Degradation of H_2O_2 produced during reaction time should be minimized until peroxidase and luminescence substrates are added. Diamine oxidase has a rather slow, but long-lasting activity. To obtain reliable signals with low concentrations of DAO, incubation has to be extended to at least 1 h. As demonstrated in Fig. 6, shaking of the reaction mixture results in a dramatic decrease of signal because of the deterioration of H_2O_2. Although the working temperature of native porcine DAO in kidney is about 37 °C, maximal signal could be achieved at room temperature. Hydrogen peroxide is obviously not stable at higher temperatures. At 4 °C, enzyme activity is not sufficient; even after 3 h reaction time, the maximum signal could not be reached.

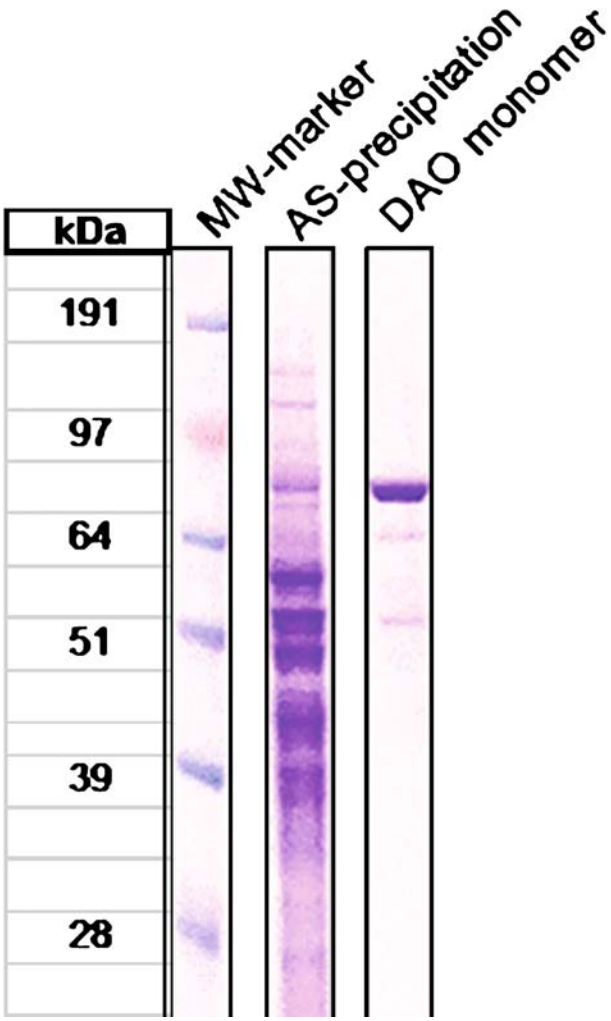


Fig. 4 Purity of DAO after the last chromatography step

As shown in Fig. 7, maximum signal intensity is achieved after 7 h of incubation time. The maximum sensitivity of the system is already reached after 5 h of incubation time. After this period, background signal increases more quickly than the specific signal related to DAO activity. Under these assay conditions, a sensitivity of purified porcine DAO of 2 U/ml (blank +2 SD) is achieved. The sensitivity of the system is not increased by incubation times longer than 5 h.

This assay allows to determine the activity of highly purified porcine DAO in a buffer system. Based on this system, we developed an assay useful for clinical use. To purify DAO from a protein solution or plasma, we used a specific antibody coated on the microtiter well.

Production of DAO-Specific Antibody

Immunization of chicken was performed successfully with the purified enzyme (see above). IgY were purified using fractionated precipitation with caprylic acid and ethanol. Purified

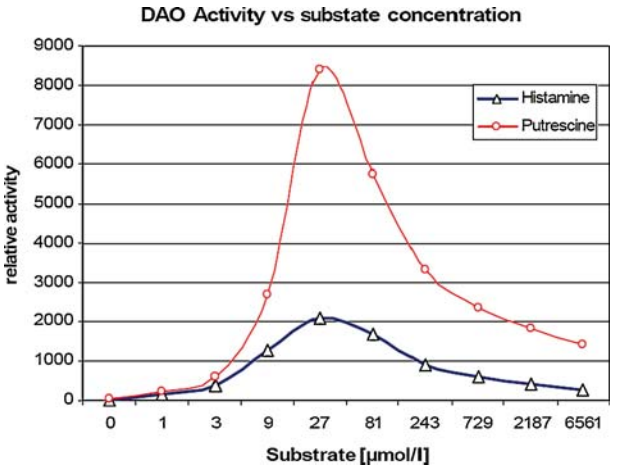


Fig. 5 Reactivity of DAO with histamine and putrescine

DAO was coated on NUNC Maxisorp microwell plates (1:0.3:1 µg/ml in 50 µM Na_2CO_3 pH 9.6; 200 µl per well, coating overnight at 4 °C). After blocking with 0.1% BSA in the same buffer system, IgY preparation was incubated overnight at 4 °C in a serial dilution. Detection of specifically bound IgY was performed with rabbit anti-IgY-AP (Aves-Labs, USA). A highly specific titer of >1:20,000 could be found for DAO, as can be seen in Fig. 8.

IgY were further purified by an affinity chromatography column (HiTrap NHS-activated; Amersham Biosciences) loaded with DAO according to the manufacturers' protocol. 500 ng of the affinity-purified antibody per well was coated on NUNC Maxisorp microtiter wells to capture DAO out of different sample matrices. Strips were blocked as described before.

Measurement of DAO Activity after Binding on IgY

To determine any inhibition of the DAO activity by antibody binding to the active site of the enzyme, increasing concentrations of the purified antibody were added to the enzyme

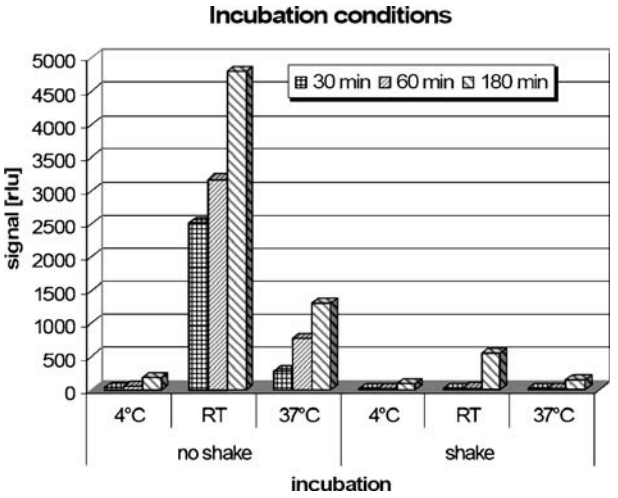


Fig. 6 Optimization of incubation conditions

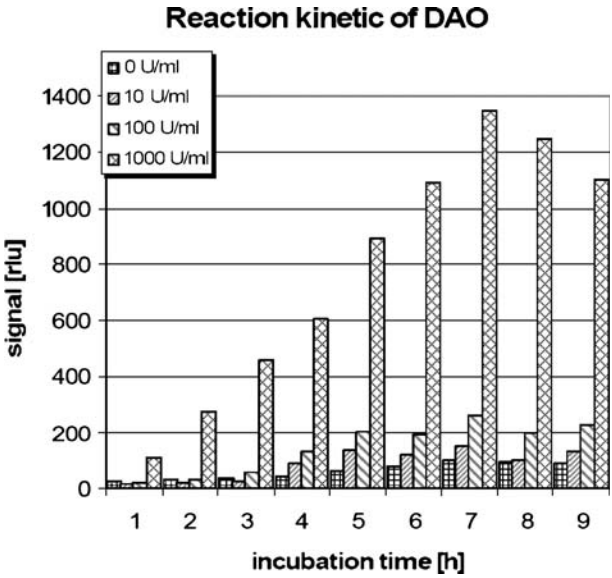


Fig. 7 Reaction kinetics of DAO

solution. Activity of the resulting enzyme–antibody complex was determined with the radioextraction assay from SCIOTEC [24]. No decrease of activity was found up to an antibody concentration of 1 mg/ml.

Binding of DAO onto the antibody occurs very slowly; therefore, incubation overnight at 4 °C has given the best results.

To measure DAO activity, 200 μl of the sample was incubated overnight on IgY-coated wells before washing the wells five times with washing buffer (WPL). Then 200 μl of putrescine solution (2.5 nmol/well diluted with 50 mM Tris pH 8) was added. For highest sensitivity, 5 h incubation is optimum. Substrate conversion was quantified by measurement

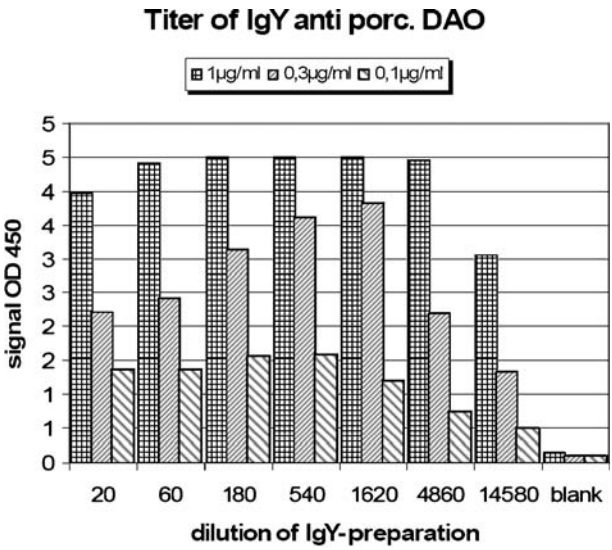
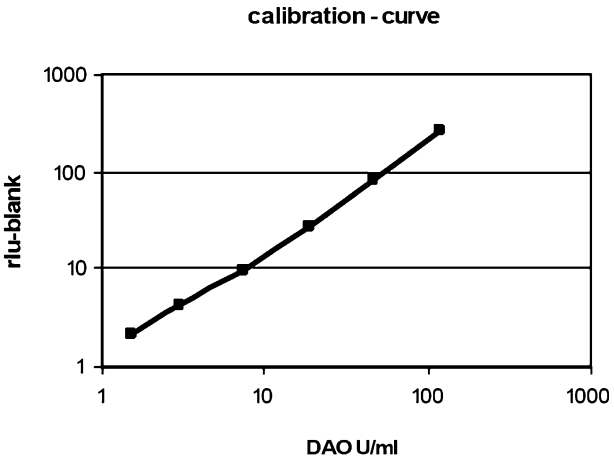


Fig. 8 Titer determination of IgY anti-DAO

Fig. 9 Typical calibration curve

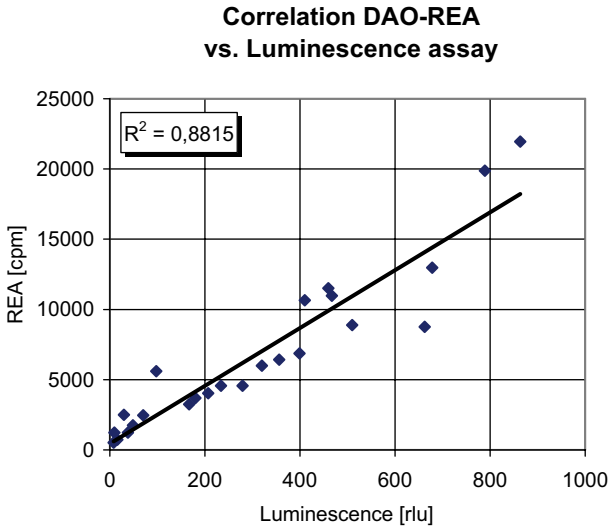


of luminescence after the addition of 20 µl PSatto™ and 50 µl horseradish peroxidase solution by a dispenser. For optimal sensitivity, the luminescence integral of the first 5 s was detected.

After isolation by antibody, the luminescence signal correlates with DAO activity of the sample, quantified by the SCIOTEC radioextraction assay. As shown in Fig. 9, it is possible to measure a few DAO U/ml. The working range of this assay is between 2 and 250 DAO U/ml, slightly better than the radioextraction assay from SCIOTEC with measuring range from 1.5 to 150 U/ml. Based on clinical data collected until now, a DAO activity of more than 15 U/ml represents a healthy person. Activities below 15 U/ml are to be considered potentially histamine-intolerant.

Using a set of 24 plasma samples, a good correlation to the existing REA test system could be shown (Fig. 10). Therefore, this assay system is a useful alternative to the existing systems. Until now, no clinical data are available for DAO activities in tissues or other biological fluids.

Fig. 10 Assay correlation with 24 plasma samples



Discussion

Up to now, no highly sensitive nonradioactive method for the determination of porcine DAO was described. The aim of this work was to develop a very sensitive and simple assay for research groups working with porcine DAO. Comparing different detection systems for hydrogen peroxide, the highly sensitive luminescence reagent PSatto™, performed best and attained the required sensitivity for hydrogen peroxide. The detection limit of PSatto™ for hydrogen peroxide, after verification by different experiments, was sufficient for the quantification of a 3 DAO U/ml.

DAO was captured from the sample by means of immunoaffinity-purified specific antibodies raised in hen using highly purified enzyme for the immunization. These antibodies were coated on microtiter wells for capturing DAO from the sample. Variations of substrate, substrate concentration, incubation time, and temperature were made to identify the best conditions for DAO.

Compared to histamine, putrescine generates a higher signal, this finding agrees with prior studies [17]. Substrate inhibition starts from 30 μM with putrescine and with histamine; hence, 25 μM putrescine seemed to be the best condition. Because of the very slow binding of DAO onto the antibody, an incubation overnight at 4 °C is preferable.

The final working protocol provides a less labor-intensive method to determine porcine DAO activity. The assay allows herewith to measure a huge amount of samples simultaneous. This fact facilitates enormous testing of inhibitors and activators of DAO.

Because of the high relevance of DAO in connection with histamine intolerance, a lot of studies are necessary to characterize the clinical pattern of enzyme inhibition.

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