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Zymographic assay of plant diamine oxidase on entrapped peroxidase polyacrylamide gel electrophoresis. A study of stability to proteolysis

Carmen Calinescu · Rodolfo Federico · Bruno Mondovi · Mircea Alexandru Mateescu

Abstract A zymographic assay of diamine oxidase (DAO, histaminase, EC 1.4.3.6), based on a coupled peroxidase reaction, and its behavior at proteolysis in simulated gastric and intestinal conditions, are described. The DAO activity from a vegetal extract of *Lathyrus sativus* seedlings was directly determined on sodium dodecyl sulfate polyacrylamide electrophoretic gels containing entrapped horseradish peroxidase, with putrescine as substrate of histaminase and *ortho*-phenylenediamine as co-substrate of peroxidase. The accumulation of azo-aniline, as peroxidase-catalyzed oxidation product, led to well-defined yellow-brown bands on gels, with intensities corresponding to the enzymatic activity of DAO. After image analysis of gels, a linear dependency of DAO content (Coomassie-stained protein bands) and of its enzymatic activity (zymographic bands) with the concentration of the vegetal extract was obtained. In simulated gastric conditions (pH 1.2, 37 °C), the DAO from the vegetal extract lost its enzymatic activity before 15 min of incubation, either in the presence or absence of pepsin. The protein pattern (Coomassie-stained) revealed that the DAO content from the vegetal extract was kept almost constant in the simulated intestinal fluid (containing pancreatin or not), with a slight diminution in the presence of pancreatic proteases. After 10 h of incubation at 37 °C, the DAO enzymatic activity from the vegetal extract was 44.7% in media without pancreatin and 13.6% in the presence of pancreatin, whereas the purified DAO retained only 4.65% of its initial enzymatic activity in the presence of pancreatin.

Keywords Diamine oxidase · Zymographic assay · Entrapped peroxidase polyacrylamide gel · Proteolytic stability · Simulated gastro-intestinal conditions

Introduction

Plant diamine oxidases (DAOs), also referred as histaminases [1], are homodimeric copper amine oxidases (EC 1.4.3.6), each subunit containing a single copper ion and 2,4,5-trihydroxyphenylalanine quinone/TPQ, a cofactor derived from the post-translational oxidation of a tyrosine residue [2]. They present a high specificity for primary diamines, able to oxidize biogenic amines to corresponding aldehyde, ammonia (NH_3), and hydrogen peroxide (H_2O_2). Current DAO assays in solution measure the amine oxidase activity by spectrophotometrical methods, monitoring directly the absorbance of formed aldehydes [3] or by subsequent condensation of different compounds [4]. Other methods are based on radiometric assays, with $[1,4-^{14}C]$ putrescine as substrate [5], on oxymetric or polarographic methods measuring the rate of oxygen consumption in the presence of substrate [6] or on fluorimetric determinations [7], where homovanillic acid is converted into a highly fluorescent compound by the released H_2O_2 in the presence of peroxidase. All these methods are not giving information

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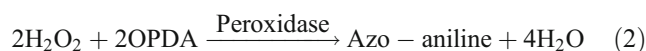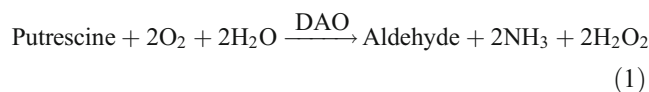Department of Biology, 3rd University of Rome,
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on the loss of molecular integrity of DAO (i.e., to acidic or proteolytic hydrolysis). Thus, supplemental information can be obtained using polyacrylamide gel electrophoresis (PAGE) by monitoring the protein pattern (staining gels with Coomassie Blue) and the enzymatic activity (zymography).

There are several major advantages of polyacrylamide (PAA) gels, such as: the high homogeneity of the gels with the density which can be easily modified to allow the best enzymes separation. The gels can be stained with Coomassie Brilliant Blue for protein profile and, keeping the same running conditions, a zymographic pattern can be directly visualized in some specific conditions and the enzyme activity can be quantified by densitometry. The zymographic PAGE is easy to run and the results are highly reproducible. As the H_2O_2 is the product of almost all oxidases, the gel areas occupied by DAO after its electrophoretic separation can be visualized via a coupled peroxidase reaction. Peroxidase as second enzyme is widely used to detect oxidase-producing H_2O_2 in presence of oxidizable dyes as its second substrate. Frequently used as donor substrate of peroxidase are: tetramethyl benzidine [8], 3,3′-diaminobenzidine [9], *ortho*-dianisidine [10], and guaiacol [11]. The chromogenic guaiacol method is one of the most commonly used on gels. However, this method has the disadvantage that the bands are stable only for a short period, requiring stabilization by Coomassie Brilliant Blue [11]. Similarly, the 3,3′-diaminobenzidine may also be used as a peroxidase chromogenic substrate, but developed bands have to be intensified by other treatments [9].

Some previous reports concern amineoxidases staining on PAA gels. Thus, Houen and Leonardsen [12] developed a specific staining method for DAO activity in the presence of peroxidase. The diaminobenzidine inhibited DAO and gave rise to unspecific staining, the most suitable among the substrates used being 4-Cl-1-naphtol. Lee et al. [13] used a peroxidase-coupled reaction in the presence of 3-amino-9-ethylcarbazole to detect plasma amineoxidase activities after native polyacrylamide gel electrophoresis. In the mentioned studies, after the electrophoresis, the gels were kept in the presence of peroxidase solution to detect amineoxidase enzymatic activities. This present study proposes a method to detect the DAO enzymatic activity from *Lathyrus sativus* seedlings using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with the peroxidase immobilized in the PAA gel. This method allowed the study of DAO stability to proteolysis in simulated gastro-intestinal conditions. To our knowledge, this is the first zymographic assay reported for vegetal histaminase in the presence of SDS using the second enzyme (peroxydase) immobilized in the PAA gel. In the presented method, we proposed the *ortho*-phenylenediamine (OPDA) as donor substrate of peroxidase, with the formation of a stable product, azo-aniline, easily monitored

on gels. The redox dyes, once oxidized, change in color and some of them also in solubility (from soluble when reduced to insoluble when oxidized). In our case, in the presence of H_2O_2 and peroxidase, OPDA changed from colorless to a yellow-brown compound stable and easy to detect on the gel. The two coupled enzymatic reactions to monitor the enzymatic activity of DAO on the SDS polyacrylamide gel containing entrapped peroxidase are:

$$\text{Putrescine} + 2O_2 + 2H_2O \xrightarrow{\text{DAO}} \text{Aldehyde} + 2NH_3 + 2H_2O_2 \tag{1}$$

$$2H_2O_2 + 2\text{OPDA} \xrightarrow{\text{Peroxidase}} \text{Azo} - \text{aniline} + 4H_2O \tag{2}$$

In our approach, the peroxidase is entrapped into the SDS polyacrylamide gels during polymerization and the DAO samples are deposited onto the gels for electrophoresis. After the electrophoretic run, gels are placed in solutions of putrescine (DAO substrate) and OPDA (peroxidase substrate). Both substrates diffuse into the gel and yellow-brown bands are developed *in situ*, corresponding to enzymatic activity of DAO.

A histaminase (DAO) of vegetal origin, more efficient than that of animal origin, was proposed for the general treatment of histamine-related pathologic conditions, such as allergic and septic shock, allergic asthma, anaphylaxis, allergic rhinitis and conjunctivitis, urticaria and atopic dermatitis, in which the histamine is the principal chemical mediator [14]. Plant histaminase can be obtained from different vegetal sources and can be used as a crude extract or as a purified enzyme. As previously shown with other copper oxidases (such as ceruloplasmin and serum bovine amine oxidase), which presented antioxidant, cardiomodulatory, and cardioprotective effects [15], vegetal DAO have beneficial effects in cardiac anaphylactic response [16] and in myocardial ischemia and reperfusion injury [17]. Plant histaminase has also some beneficial effects in asthma-like reaction [18], or in splanchnic artery occlusion/reperfusion injury [19]. As histamine and reactive oxygen species are involved in the pathophysiology of inflammatory bowel disease, hog kidney DAO had been intraperitoneally administered on experimental ulcerative colitis in rats [20], and the DAO treatment positively modified the inflammatory reaction. Thus, it is also expected that exogenous DAO could protect against oxidative damage [17–19], as reactive species also play an important role in inflammatory diseases. In this context, the vegetal DAO stability to proteolysis in the presence of digestive enzymes has to be known. A proteolysis study on a vegetal DAO purified from pea (*Pisum sativum*) seedlings was previously reported [21] in the presence of 0.01% pepsin (pH 2) and of

0.1% trypsin (pH 7.2). To our knowledge, there are no studies on vegetal histaminase in more acidic conditions (pH 1.2) and over longer periods in simulated intestinal fluid, in the presence of pancreatin (containing trypsin and other proteases, together with other various digestive enzymes such as amylase, lipase, ribonuclease) [22]. In this context, the aim of this work was also to study *in vitro* the behavior of DAO from *L. sativus* seedlings extract to proteolytic action of digestive enzymes, in simulated gastric and intestinal conditions, using the zymographic assay described above.

Experimental

Materials

1,4-Diaminobutane dihydrochloride (putrescine), *ortho*-phenylenediamine dihydrochloride, peroxidase type I (from horseradish, 96 purpurogallin units/mg solid), Bradford Reagent, pepsin (from porcine gastric mucosa, 882 units/mg protein), and pancreatin (from porcine pancreas) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Acrylamide, *N,N′*-methylene-bis-acrylamide and protein molecular weight standards (Broad Range) were from Bio-Rad Laboratory (Richmond, VA, USA).

Preparation of vegetal extract from *L. sativus* seedlings and purification of DAO

The vegetal extract and the purified DAO from grass pea *L. sativus* seedlings were prepared as previously described [23], with minor modifications. Briefly, 500 g of freshly collected shoots of etiolated *L. sativus* seedlings were homogenized in a Waring blender with 1 L of 30 mM NaH_2PO_4 (final pH 4.4), and then filtered. In these conditions, the DAO remains ionically linked to the insoluble fraction. The solid residue, mainly constituted by cell walls and vascular fibers, was washed with the same buffer and the enzyme was finally eluted from the solid residue with 500 mL of 0.1 M sodium phosphate buffer (pH 7) and, then, centrifuged. The supernatant containing the DAO was lyophilized. The purification of DAO was done as previously described [23], onto a DE52-cellulose and a HiTrap SP Sepharose column.

Determination of protein concentration and of enzymatic activity of DAO preparations from *L. sativus* seedlings

Different concentrations of vegetal extract (1, 5, 10, 15, 25, 40 mg/mL) or of purified DAO (3 mg/mL) were kept for 2 h at 4 °C in phosphate buffer solution (PBS, pH 7.4)

under agitation and then, filtered and rapidly frozen. Protein concentrations of the vegetal extract and of the purified DAO were determined by the method of Bradford [24], using bovine serum albumin as standard. The enzymatic activity of DAO was assayed spectrophotometrically with the same two coupled reactions as for zymography assay, in the presence of putrescine (30 mM) as substrate for DAO and of peroxidase as a second enzyme reaction, where OPDA is enzymatically oxidized into a colored compound (azo-aniline) by the released H_2O_2. The incubation mixture contained 640 µL of PBS (10 mM sodium phosphate buffer, pH 7.4), 10 µL of peroxidase solution (0.1 mg/mL), 50 µL of OPDA solution (30 mM), 200 µL of putrescine solution (30 mM) and 100 µL of DAO samples of unknown concentrations. The mixtures containing PBS, peroxidase, OPDA, and putrescine were incubated for 5 min at 37 °C, and then, the DAO samples were added. The enzymatic reactions were incubated at 37 °C for 10 min, when 100 µL of HCl (4 M) were added and the final absorbance was read at 484 nm using a Beckman DU®-6 spectrophotometer. The standard curve was prepared with serial concentrations of H_2O_2 from 0 to 68 µM.

One enzymatic unit (EU) of DAO is defined as the amount of enzyme catalyzing the oxidation of 1.0 µmole of putrescine per 10 min at pH 7.4, at 37 °C.

Proteolysis of DAO

DAO was incubated in simulated gastro-intestinal conditions, with and without pepsin in simulated gastric fluid (SGF) or with and without pancreatin in simulated intestinal fluid (SIF) [22]. Samples of 40 mg of vegetal extract powder were each incubated for 0, 5, 10, 15, 30, 60 and 120 min in 875 µL SGF (pH 1.2), containing or not pepsin (0.32% powder with 882 units/mg protein). In parallel, the same amounts of vegetal extract (40 mg) were each incubated for 0, 1, 2, 4, 6, 8, and 10 h in 1 mL SIF (pH 6.8), containing or not pancreatin. The SIF containing pancreatin only was also incubated (as control) for the same periods of time as above. The incubations were done at 37 °C and 50 rpm using an incubator shaker (series 25D, New Brunswick Scientific Co., New Jersey, USA).

After each indicated SGF period, every SGF sample was neutralized with 125 µL of 6.66% sodium bicarbonate solution and maintained under agitation for 2 h at 4 °C. Then, the neutralized SGF samples (final concentration, 40 mg/mL) were filtered and frozen. The SIF samples were only filtered and rapidly frozen. Purified DAO (3 mg/mL) was incubated only for 10 h in SIF containing pancreatin (pH 6.8, 37 °C and 50 rpm), then filtered and rapidly frozen.

The DAO standards were represented by the vegetal extract (40 mg/mL) or by the purified DAO (3 mg/mL) in

PBS solution (pH 7.4). Both standards were kept for 2 h under agitation at 4 °C and then filtered and frozen.

All the samples of histaminase were run in SDS-PAGE under non-reducing conditions.

Peroxidase entrapment in polyacrylamide gels

For the enzymatic detection of DAO on gels via the peroxidase-coupled reaction, the peroxidase was entrapped in the PAA gels. Thus, during the 8% PAA resolving gels preparation, 1 mL of horseradish peroxidase (1 mg/mL) was added to the gel solutions prior to polymerization (final volume of 5 mL). Stacking gels contained no peroxidase. To verify the homogenous distribution of peroxidase in the polymerized gel, an 8% PAA resolving gel containing peroxidase (19.2 purpurogallin units) was electrophoretically tested for 1 h (room temperature, 120 V) with no samples loaded on it. Then, the gel was immersed in a staining solution containing equal volumes of H_2O_2 (30 mM) and OPDA (30 mM) and kept under weak agitation for 1 h. Another 8% PAA resolving gel, containing no entrapped peroxidase and no samples loaded on it was treated in the same conditions (control).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

The DAO protein content and the enzymatic activity of the vegetal samples incubated in PBS or in simulated gastric and intestinal conditions were evaluated by SDS-PAGE, using the electrophoresis system Mini-Protean II (Bio-Rad). Each sample was run in two PAA gels: one with entrapped peroxidase for zymography and another peroxidase-free for protein pattern.

Thus, the samples of vegetal extract at different concentrations in PBS (1, 5, 10, 15, 25, 40 mg/mL), the neutralized SGF samples (40 mg vegetal extract/mL in SGF, with or without pepsin), the SIF samples (40 mg vegetal extract/mL in SIF, with or without pancreatin) and the samples of purified DAO (3 mg/mL in SIF with pancreatin), prepared as described above, were defrosted and treated (1:1, v/v) with SDS electrophoresis sample buffer containing 0.12 M Tris–HCl (pH 6.8), 4% SDS (138 mM), 20% glycerol, 0.004% bromophenol blue and no beta-mercaptoethanol. The mixtures were not heated. Then, 30 µL of each treated sample were loaded and resolved by SDS-PAGE for 1 h and 15 min (room temperature, 120 V) on 8% PAA peroxidase-free gels for Coomassie Blue staining of proteins or on 8% PAA gels containing entrapped peroxidase (19.2 purpurogallin units) for zymographic revelation of DAO enzymatic activity. The electrophoresis buffer used in electrophoresis runs contained 0.025 M Tris–Base, 0.192 M glycine, and 0.1% SDS.

The molecular weight protein standards were diluted in SDS reducing sample buffer (with beta-mercaptoethanol), heated for 5 min at 95 °C, then cooled and loaded 10 µL/well to the PAA gel, as indicated in specifications from Bio-Rad.

Coomassie Blue protein staining

After electrophoresis, the 8% PAA gels were incubated for 30 min (mild agitation) in a fixation solution containing methanol:acetic acid:water (50/10/40, $v/v/v$) followed by 1 h of staining with 0.5% Coomassie Brilliant Blue G-250 in a methanol:acetic acid:water (40/10/50, $v/v/v$) solution.

Detection of DAO enzymatic activity on polyacrylamide gels

After electrophoresis, the PAA gels containing entrapped peroxidase and electrophoretically separated DAO samples, were rinsed with distilled water and placed in a solution of putrescine (30 mM) for a few minutes. Then, the incubation medium was completed with an equal volume of OPDA solution (17 mM). The DAO enzymatic activity was detected on PAA gels (zymographic bands) in function of newly produced substrate (H_2O_2) and in saturating concentration of chromogenic substrate (OPDA). Each substrate solution was prepared in PBS (pH 7.4) and kept at 4 °C before utilization. The gels immersed in the two mentioned solutions were incubated at 37 °C (1 h) under dark conditions and weak agitation. The position of DAO enzymatic activity bands on gels (zymograms) was determined by comparison of the electrophoretic patterns obtained from Coomassie staining, using DAO in PBS solution (pH 7.4) as standard. The densitometry image analysis of gel bands was carried out using the *Quantity One* program (Bio-Rad). The DAO enzymatic activity measured by densitometry of bands was correlated with the DAO initial enzymatic activity (DAO in PBS, pH 7.4), spectrophotometrically determined. The DAO percentages were reported to standard, considering the standard (DAO in PBS, pH 7.4) as 100%.

Influence of SDS on the enzymatic activity of DAO

The estimation of the DAO enzymatic activities monitored on the zymographic gels (EU/electrophoretic load of 30 µL, in the presence and the absence of SDS) and the dependence of DAO specific enzymatic activity on the time of incubation with SDS was studied spectrophotometrically at 25 °C, in the same conditions as for electrophoresis. Thus, samples of vegetal extract in PBS (1, 5, 10, 15, 25, 40 mg/mL) were treated (1:1, v/v) with an electrophoresis buffer containing 0.12 M Tris–HCl (pH 6.8), 20%

glycerol, in the presence or the absence of 4% SDS (138 mM), for the determination of the DAO enzymatic activities as EU/electrophoretic load of 30 μL. The dependence of DAO specific enzymatic activity on the time of incubation was studied after 0, 30, 75, and 135 min at 25 °C, on a sample of 40 mg vegetal extract/mL, in the same electrophoresis buffer and in the presence or in the absence of SDS. The DAO enzymatic activities were determined by the same spectrophotometrical method described above, using the two coupled reactions in solution.

Results and discussion

The protein content of *L. sativus* vegetal extract, determined by Bradford assay [24], was in a linear dependency ($R^2=0.9978$) with the vegetal extract concentrations (1, 5, 10, 15, 25, 40 mg/mL), indicating a good homogeneity of the extract powder (data not shown). The same concentrations of vegetal extract as mentioned above (with a total protein content of 15 ± 1.4 μg/mL, 132 ± 8 μg/mL, 302 ± 13 μg/mL, 435 ± 17 μg/mL, 719 ± 33 μg/mL and, respectively, $1,091\pm27$ μg/mL) were then used on electrophoretic gels to follow the DAO protein content (Coomassie coloration) and the DAO enzymatic activity (zymography). The same migration patterns of molecular weight standards and of vegetal extract were obtained for either gels containing or not entrapped peroxidase after Coomassie coloration (data not shown). Thus, throughout the whole study, we used gels containing entrapped peroxidase only for the enzymatic activity evaluation of DAO (zymography).

To our knowledge, this is the first zymographic assay for plant histaminase with the second enzyme (peroxidase)

Fig. 1 Electrophoretic pattern of vegetal extract from *L. sativus* seedlings. Coomassie staining: **a** SDS-PAGE (8% PAA resolving gel without peroxidase) of different concentrations of DAO vegetal extract (1, 5, 10, 15, 25, 40 mg powder/mL), treated (1:1, *v/v*) with non-reducing SDS loading buffer (30 μL/well). The gels were stained with Coomassie Brilliant Blue G-250. **b** Image analysis of DAO bands intensity with the *Quantity One* program. $n=3$. Zymographic DAO enzymatic activities: **c** different concentrations of DAO vegetal extract (1, 5, 10, 15, 25, 40 mg powder/mL), treated (1:1, *v/v*) with non-reducing SDS loading buffer (30 μL/well). Oxidase activity was directly monitored on 8% PAA gels (containing entrapped peroxidase) after the electrophoretic run, in the presence of putrescine and *ortho*-phenylenediamine. **d** Image analysis of DAO bands intensity (enzymatic activity) with the *Quantity One* program. **e** Enzymatic activities of DAO on gel (EU/electrophoretic load of 30 μL), spectrophotometrically determined, in function of the vegetal extract concentrations. Protein content of vegetal extract: 15 ± 1.4 μg/mL (1 mg/mL), 132 ± 8 μg/mL (5 mg/mL), 302 ± 13 μg/mL (10 mg/mL), 435 ± 17 μg/mL (15 mg/mL), 719 ± 33 μg/mL (25 mg/mL), $1,091\pm 27$ μg/mL (40 mg/mL). $n=3$
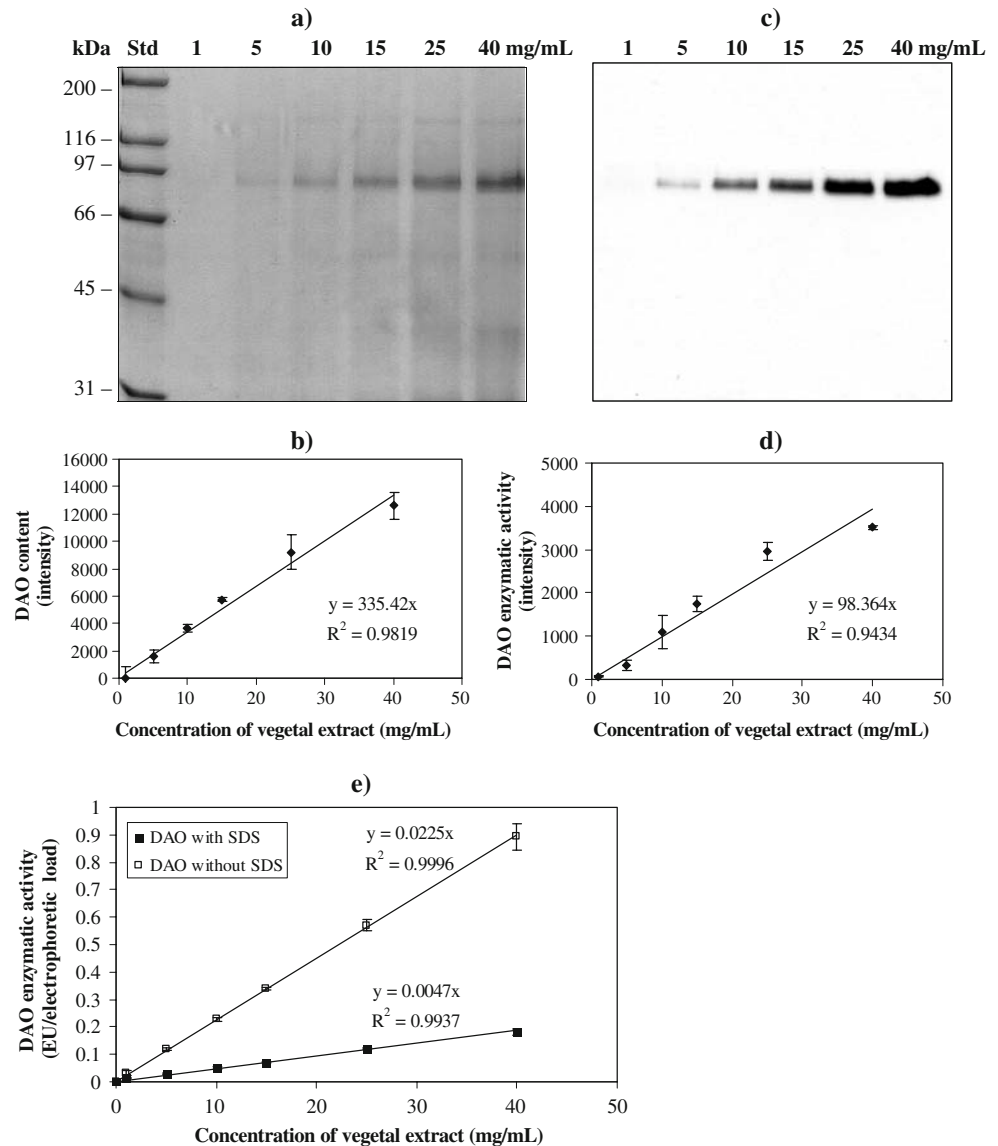
entrapped in the PAA gel. As control, after an electrophoresis run without any DAO-loaded samples, the PAA resolving gel, containing entrapped peroxidase, was colored in a few minutes and in a homogenous manner in the presence of H_2O_2 and OPDA solutions. This indicated that the entrapped peroxidase still kept its enzymatic activity and that it was uniformly distributed in the PAA resolving gel. The gel without entrapped peroxidase, kept under the same conditions as above, remained totally transparent even after 1 h of incubation (data not shown). There are lots of applications with peroxidase physically entrapped in gels. In our case, the enzyme was immobilized during polymerization of PAA gels (a covalent immobilization during the polymerization process of acrylamide gels involving double bonds of the prosthetic group of peroxidase is not excluded). Thus, despite the molecular weight of peroxidase (40 kDa), much lower than that of DAO from *L. sativus* (approximately 150 kDa), peroxidase did not migrate during the electrophoresis run, suggesting its immobilization into the PAA gel during the polymerization process, a fact also reported by Ugarova et al. [25], who studied the catalytic properties and stability of immobilized horseradish peroxidase into a PAA gel.

It is known that in denaturing SDS-PAGE, migration of proteins is determined primarily on their molecular mass. A single band in reducing SDS-PAGE corresponding to a molecular mass of 72 kDa [23] was reported for DAO from *L. sativus*. In non-reducing SDS-PAGE (without beta-mercaptoethanol and without heating of samples, which both can disrupt the folded structure of enzyme), the electrophoretic mobility of proteins depends more on their hydrodynamic size. Thus, the SDS-PAGE pattern of the analyzed vegetal extract presented a major protein band, identified as DAO, whose growing intensity was in function of the concentration of the loaded vegetal extract (Fig. 1a). Gel images were densitometrically analyzed using the *Quantity One* program and a linear variation of histaminase content (protein band intensity) with the concentrations of vegetal extract was obtained (Fig. 1b). The combination of SDS-PAGE protein pattern (Coomassie staining) and of zymogram technique allowed to determine comparatively the position of zymographic bands corresponding to DAO enzymatic activity (Fig. 1c). The H_2O_2, the product of enzyme reaction of DAO and substrate for peroxidase, will generate, in the presence of OPDA dye (second substrate for peroxidase), a colored oxidized product (azo-aniline) with intensity related to the amount of released H_2O_2 resulted from DAO enzyme activity. The oxidized product of the two coupled enzymatic reactions is thus constantly accumulated on the gel. In all cases, the zymograms revealed only a single yellow-brown band on a colorless background, attributed to the DAO activity of histaminase, with intensity of bands

growing in function of the concentration of vegetal extract, showing a linear dependency (Fig. 1d). Normally, the SDS contributes to unfold an enzyme by interacting with the non-polar amino acid side-chains situated in the interior of the folded protein structure. In our non-reducing conditions (without beta-mercaptoethanol and without heating of samples), the DAO still retained its enzymatic activity in the presence of SDS, a linear dependency being obtained between DAO enzymatic activity (EU/electrophoretic load) and the concentrations of the vegetal extract, either in the presence or the absence of SDS (Fig. 1e).

The SDS is known to interact with most of water soluble proteins to form protein–detergent complexes [26]. Many enzymes lose their activity in the presence of SDS and some are activated [27, 28]. Gebicka [29] showed that horseradish peroxidase in solution is relatively resistant against SDS, the enzyme retaining its full activity up to 20 min when incubated at room temperature with 100 mM SDS, and still retained 63% of its activity after the next 4 h. To our knowledge, there are no studies on vegetal histaminase in the presence of SDS. Since in our case, it was observed that DAO still retained part of its enzymatic activity on PAA gels in the presence of SDS (Fig. 1c–e), it was of interest to study the stability of vegetal DAO in solution in the presence of SDS, using the same conditions as for electrophoresis. Thus, in the absence of SDS, the enzymatic activity of DAO was kept constant during the first 30 min of incubation (25 °C), and then, its activity significantly diminished (Fig. 2). In the presence of 69 mM SDS, the DAO specific activity was rapidly reduced, but then being stabilized for the entire period of 135 min of
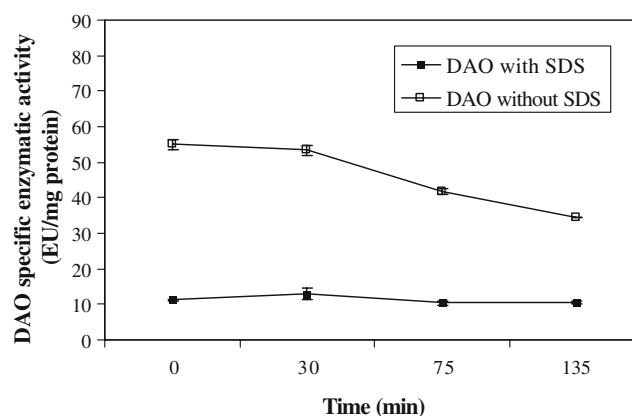

Fig. 2 The influence of sodium dodecyl sulfate on the enzymatic activity of DAO. The specific enzymatic activity of DAO (EU/mg protein) from vegetal extract (40 mg/mL, containing 1,091±27 μg total protein/mL) was measured at 25 °C in a solution containing 60 mM Tris–HCl (pH 6.8), 10% glycerol, in the presence or the absence of 69 mM SDS. The DAO specific enzymatic activities were determined spectrophotometrically, as described before, with putrescine as DAO substrate and *ortho*-phenylenediamine as co-substrate for peroxidase; $n=3$

incubation in SDS (Fig. 2). This stabilization of the enzymatic activity in the presence of SDS for more than 2 h is important, since the revelation of enzymatic activity of DAO on PAA gels was done after 75 min of electrophoresis and during 60 min of gel incubation. The decrease of DAO enzymatic activity by SDS was also settled as percentage from the original DAO specific activity (in the absence of SDS), which was considered as 100%. Thus, 30% of DAO enzymatic activity was still detected after 135 min of incubation in the presence of SDS. The presence of SDS in the electrophoresis conditions (SDS-PAGE) contributed to a better resolution of DAO separation comparatively to native PAGE (data not shown), improving mobility and stabilizing the DAO.

The behavior of DAO from *L. sativus* seedlings extract (40 mg vegetal extract/mL containing 1,091±27 μg total protein/mL) under proteolytic action of digestive enzymes was studied in SGF (with or without pepsin) and in SIF (with or without pancreatin) [22]. Thus, DAO electrophoretic bands corresponding to its protein content and its enzymatic activity were revealed on electrophoretic gels for DAO in PBS (pH 7.4) and in neutralized SGF (with or without pepsin), at 0 min of SGF treatment (Fig. 3). In acidic conditions (pH 1.2), the degradation and inactivation of DAO (with no detectable DAO enzymatic activity on gels) were observed after 15 min of incubation at 37 °C and 50 rpm (Fig. 3a, b). The pH of an enzyme medium can affect the state of ionization of amino acid side-chains that

Fig. 3 Electrophoretic pattern of diamine oxidase from the vegetal extract of *L. sativus* seedlings in simulated gastric fluid (SGF, pH 1.2). SGF without pepsin (**a**, **b**) and SGF with pepsin (**c**, **d**). Coomassie staining gels without peroxidase (**a**, **c**) and DAO zymographic gels containing entrapped peroxidase (**b**, **d**) of vegetal extract (40 mg/mL, containing 1,091± 27 μg total protein/mL) from *L. sativus* seedlings in PBS, pH 7.4 and in SGF (up to 120 min of incubation). The vegetal extract samples in SGF (with or without pepsin) were neutralized with sodium bicarbonate, and then treated with non-reducing SDS loading buffer (1:1, *v/v*) and loaded 30 μL/well. DAO protein content (**e**) and DAO enzymatic activity (**f**) in SGF. Results are expressed in percentage, considering DAO in PBS as 100%. *Quantity One* program was used for densitometric analysis of gel images. The DAO specific enzymatic activity of 11.21± 0.31 EU/mg protein, determined spectrophotometrically in PBS solution (in the presence of SDS), was considered as 100%; *n*=3
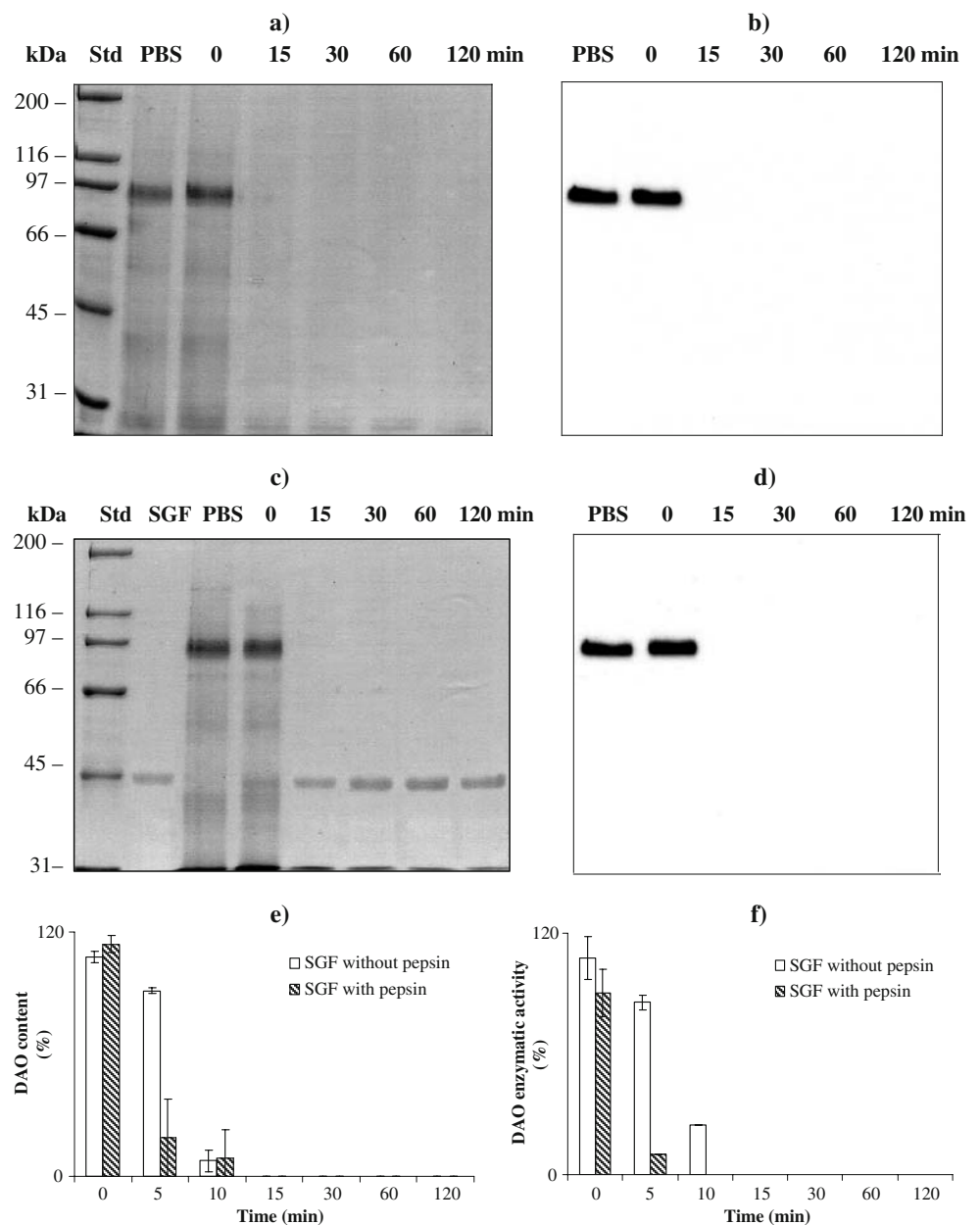
may be involved in the maintenance of the folded structure. Furthermore, in strong acidic conditions (pH 1.2), a possible hydrolysis can also occur with the degradation of the proteins contained in the vegetal extract (Fig. 3a). Federico et al. [21] reported that at pH 2 and in the presence of pepsin (0.01%, 4,500 units/mg), purified histaminase from pea seedlings (*P. sativum*) was partially inactivated after 30 min of incubation (14% remaining enzymatic activity), with complete loss of activity after 60 min of incubation. In more acidic conditions (pH 1.2) and in the presence of pepsin (0.32% with 882 units/mg protein) [22], the total degradation of all the proteins present in the vegetal extract from *L. sativus* seedlings was observed after 15 min of incubation (Fig. 3c), with no detectable DAO enzymatic activity (Fig. 3d). The remaining band found for samples incubated at 15, 30, 60, and 120 min corresponds to pepsin which is stable in gastric acidity (Fig. 3c).

The time course in SGF, in the absence or in the presence of pepsin, indicated a fast degradation of DAO in early stage of incubation (Fig. 3e,f). Thus, after 5 min of acidic incubation, a loss of about 10% of DAO content and of 15% of DAO activity detected on gels was observed. At 10 min, the degradation of DAO was more advanced, and, after 15 min, its degradation was complete. In the presence of pepsin, the DAO degradation was even faster, with total loss of its enzymatic activity in less than 10 min.

Previous proteolysis studies with other amine oxidases, such as swine kidney diamine oxidase (SKDAO) and bovine serum amine oxidase (BSAO), were done separately

with trypsin, pronase, or with thermolysin for a period of up to 4 h [30]. The SKDAO in the presence of trypsin or pronase partially lost its enzymatic activity in the first 60 min, followed by stabilization during the next 3 h of proteolysis. In the case of trypsin, the SKDAO activity was stabilized at about 50% after 4 h of hydrolysis. In the presence of thermolysin, the SKDAO lost more than 60% of its initial activity after 4 h of proteolysis. Concerning the BSAO, the pronase exerted the highest denaturing effect, with only 40% remaining enzymatic activity after 4 h of hydrolysis. There are not many studies on vegetal histaminase stability to proteolysis in the presence of pancreatic enzymes. Federico et al. [21] tested purified vegetal histaminase from *P. sativum* seedlings only for 60 min in the presence of trypsin, when 67% of its enzymatic activity was retained. In our study, pancreatin, containing enzymes such as trypsin, chymotrypsin, endopeptidases, exopeptidases, carboxypeptidases, as well as other digestive enzymes (amylase, lipase, ribonuclease), was used to test the proteolysis stability of DAO from *L. sativus* over longer periods, up to 10 h. During 10 h of vegetal extract incubation in pancreatin-free SIF (pH 6.8) at 37 °C and 50 rpm, the DAO protein content was kept constant as indicated after Coomassie Blue staining (Fig. 4a), with a slight diminution of its enzymatic activity on the corresponding zymogram (Fig. 4b). Interestingly, in the presence of pancreatin, in the same conditions as before (SIF, pH 6.8), the DAO from the vegetal extract was still present after 10 h of incubation, as indicated by Coomassie

Fig. 4 Stability of diamine oxidase from *L. sativus* vegetal extract in simulated intestinal fluid (SIF, pH 6.8). SIF without pancreatin (**a, b**) and SIF with pancreatin (**c, d**). Coomassie-stained gels without peroxidase (**a, c**) and DAO zymographic gels containing entrapped peroxidase (**b, d**) of vegetal extract (40 mg/mL, containing 1,091±27 μg total protein/mL) from *L. sativus* seedlings in PBS, pH 7.4 and in SIF (up to 10 h of incubation). The vegetal extract samples in SIF (with or without pancreatin) were treated with non-reducing SDS loading buffer (1:1, *v/v*) and loaded 30 μL/well; *n*=3
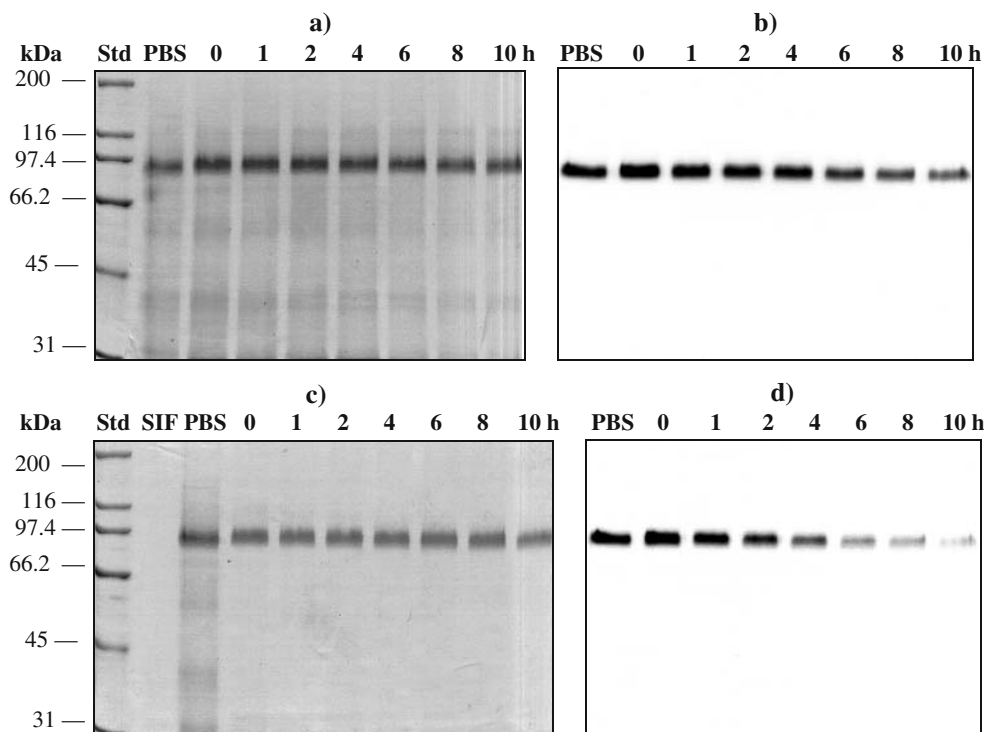
protein staining (Fig. 4c). The corresponding zymogram still showed a band ascribed to DAO enzymatic activity even after 10 h of incubation in the presence of pancreatin (Fig. 4d), indicating a certain stability of DAO to pancreatin proteolysis. As control, SIF containing pancreatin only was spectrophotometrically checked for amine oxidase, and no activity was found over the 10 h of incubation (data not shown). This confirms the lack of additional interfering amine oxidases due to eventual microbial contamination during the long incubation. The densitometric analysis (Fig. 5) confirmed that the DAO protein content was almost constant either in the absence or presence of pancreatin, with a slight decrease in the presence of pancreatin (Fig. 5a). After 10 h of incubation at 37 °C (50 rpm), the DAO activity from the vegetal extract was 44.7% in SIF

a)

b)

Fig. 5 Densitometric analysis of diamine oxidase from *L. sativus* vegetal extract after incubation in simulated intestinal fluid (SIF, pH 6.8), with or without pancreatin. **a** DAO protein content (%), **b** DAO enzymatic activity (expressed in % from the specific activity of DAO in PBS). Results are expressed in percentage, considering DAO in PBS as 100%. *Quantity One* program was used for densitometric analysis of gel images. The vegetal extract (40 mg/mL) contained 1,091±27 µg total protein/mL in PBS/SIF. The DAO specific enzymatic activity of 11.21±0.31 EU/mg protein, determined spectrophotometrically in PBS (in the presence of SDS), was considered as 100%; *n*=3

without pancreatin and 13.6% in the presence of pancreatin (Fig. 5b).

To evaluate the influence of the presence of the other proteins from the *L. sativus* vegetal extract on DAO proteolysis, a purified DAO preparation (containing 413± 9 µg protein/mL with a specific enzymatic activity of 110.43±2.01 EU/mg protein, determined in the presence of SDS) from the same vegetal source was also tested for 10 h (37 °C, 50 rpm) only in the presence of pancreatin (SIF, pH 6.8). The densitometric analysis of purified DAO protein content and of its enzymatic activity was realized, considering the purified DAO in PBS (pH 7.4) as standard (100%). After 10 h of incubation in the presence of pancreatin, the protein content of purified DAO diminished to 59.7% (data not shown), more than in the case of DAO from vegetal extract, which decreased to 78.7% after 10 h (Fig. 5a), suggesting a higher susceptibility at proteolysis of the purified histaminase samples. Concerning the DAO enzymatic activity, after 10 h of incubation in the same conditions as before, only 4.65% of purified DAO activity was still present (data not shown), compared to 13.6% obtained with the vegetal extract (Fig. 5b). These results suggest a possible protection of DAO afforded by the other proteins present in the vegetal extract or a possible presence of some protease inhibitors in the vegetal extract able to partially protect the DAO against pancreatin proteolysis. The elucidation of these aspects, explaining better stability to proteolysis in the case of the vegetal extract, will be the object of a further investigation.
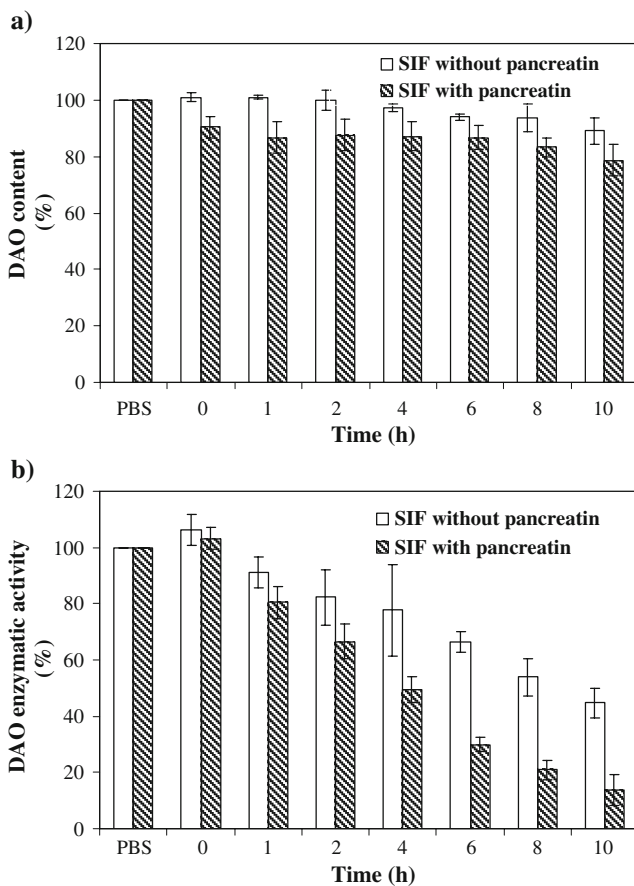
Conclusion

A zymographic method was developed for the monitoring of DAO activity on immobilized peroxidase in polyacrylamide gels, with putrescine as substrate of DAO and OPDA as second substrate for peroxidase. Combination of protein pattern obtained in SDS-PAGE with that of enzymatic activity obtained in zymography can give valuable information on protein stability and enzymatic activity of DAO. Furthermore, a correlation between the DAO content and its enzymatic activity from a vegetal extract can be established in different conditions (i.e., proteolysis). The data obtained by this method indicated a certain stability at proteolysis of vegetal DAO, an aspect which can be of interest for eventual therapeutic application of this enzyme. This method can also be used on native PAGE gels, as an alternative method to evaluate the DAO enzymatic activity.

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