Current Topics in Peptide & Protein Research
Vol. 23, 2022

Original Article

Self-mediated histaminase obtained by an environment-friendly mechanical procedure

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ABSTRACT

Diamine oxidase (DAO, histaminase) is known to reduce inflammation due to allergic response to exogeneous/endogenous amines and to be beneficial in topical applications for skin conditions. Foods, especially fermented foods, are an important source of exogeneous amines and deficient/low-activity intestinal DAO may result in increase in the histamine level in plasma and, eventually, in histamine intolerance. Additionally, improper processing/storage of foods may contribute to increase in histamine level in those foods, leading to histamine poisoning, as well as an increase in other biogenic amines' content. This makes quantitation of biogenic amines in foods important for ensuring the desired quality and freshness, especially in fish/shellfish, and because of their inhibitory effect on histaminase activity. Instrumental methods for biogenic amine detection are expensive and some require derivatization. While amperometric biosensors using DAO are sensitive and inexpensive, their drawback is the high cost and low activity of commercially available DAO. This study presents a green approach to enhance and stabilize DAO from porcine kidney using high hydraulic pressure (HHP). A mechanical procedure is presented in which DAO is compressed in the presence of enzyme-friendly redox species (Cu^{2+}, pyridoxal phosphate, PLP). DAO unfolds partially and refolds, after HHP removal. All modified DAO samples obtained retained activity. Copper ions (intrinsic to DAO) were more beneficial than PLP and shorter compression times resulted in more active enzymes. DAO-friendly redox units entrapped within the 3-D structure of histaminase afford a self-mediated, "wired" DAO which was tested in an amperometric biosensor. The catalytic effect and linearity of the response recommend this biosensor for use in detection of biogenic amines. The procedure presented is simple, avoids toxic mediators/reagents used in enzyme "wiring" and it results in DAO which is molecularly "wired" and able of direct electron transfer. The modified DAO was stable over several months and warrants testing in medical applications as well.

KEYWORDS: histaminase, diamine oxidase, "wired", self-mediated, high hydraulic pressure.

ABBREVIATIONS

AAP	-	4-aminoantipyrine
CD	–	circular dichroism
CU	–	copper II sulfate pentahydrate
DAO	–	diamine oxidase, histaminase
DAO-ME	–	processed diamine oxidase
DAO-CU	–	diamine oxidase processed in the presence of copper sulfate
DAO-PLP	–	diamine oxidase processed in the presence of pyridoxal phosphate
DCHBS	-	3,5 dichloro-2-hydroXybenzene-sulfonic acid
DET	–	direct electron transfer
FAD	–	flavin adenine dinucleotide
GC	–	gas chromatography

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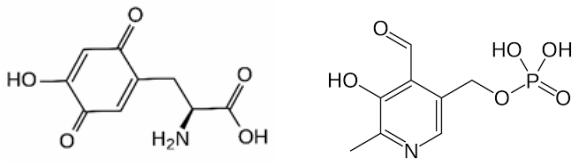
GOX – glucose oxidase
HHP – high hydraulic pressure
HPLC – high performance liquid chromatography
HRP – horseradish peroxidase
LDH – lactate dehydrogenase
MET – mediated electron transfer
PAO – plasma amine oxidase
PAO-CU – plasma amine oxidase processed in the presence of copper sulfate
PAO-PLP – plasma amine oxidase processed in the presence of pyridoxal phosphate
RTIL – room temperature ionic liquid
SCE – standard calomel electrode
TPQ – 2,4,5-trihydroxyphenylalanine quinone, topaquinone
Trp – tryptophan
WE – working electrode

1. INTRODUCTION

Diamine oxidase (DAO, histaminase) is a homodimeric copper enzyme, containing a copper II ion and a redox cofactor, 2,4,5-trihydroxyphenylalanine quinone (topaquinone, TPQ, Fig. 1) in each subunit (molecular weight of 87 kDa) [1]. It catalyzes the oxidative deamination of primary amine groups on histamine [2] and other biogenic amines (such as putrescine, cadaverine, spermine, spermidine, tryptamine, tyramine, agmatine), producing corresponding aldehydes, ammonia, and hydrogen peroxide (equation 1). These amines represent health hazards responsible for vascular and gastrointestinal problems.

$$R\text{-}CH_2\text{-}NH_2 + O_2 + H_2O \longrightarrow R\text{-}CHO + NH_3 + H_2O_2 \quad (1)$$

In the medical field, DAO has been reported to have a protective effect in cardiac patients and in ischemic bowel [3], to reduce inflammation due to allergic response to exogeneous and endogenous amines [4], and to be beneficial in topical applications for different skin conditions [5]. In humans, DAO can be found in the placenta, kidneys, and intestine [6]. The intestinal DAO protects against exogeneous amines from foods [3, 7] and, if it is deficient/has low activity, it may result in increase in the histamine level in the plasma and, eventually, in histamine intolerance [8]. Biogenic amines are important for the food industry as well since they can exist in meats, fish, dairy, and even in some vegetables [8, 9]. Fermented foods (wine, cheeses, sauerkraut, fermented meats) are important sources of biogenic amines due to the presence of microorganisms producing decarboxylases and the environment rich in proteins which favors their growth [10]. Additionally, unsafe conditions during processing and storage may contribute to the increase in the histamine level in those foods (above 500 ppm), leading to histamine poisoning, as well as an increase in other biogenic amines' content [11]. Even small quantities of histamine can trigger allergic responses in sensitive individuals. Recent studies estimate that approximately 1% of the total population suffers from histamine intolerance [12]. An approach reported to control histamine in foods is adding bacteria with histamine degradation activity to fermented foods to ensure safety [13, 14]. The fact that bacteria may alter the taste of the foods, that they may not be used for foods which are not fermented, and that they do not eliminate histamine completely limits the applicability of this procedure [13, 15]. Another reported strategy to reduce the intake of biogenic amines from foods is oral administration of DAO [16]. Commercial products like Daosin, containing DAO from porcine kidney, DAOgest, Histamine Block, or Vinpocetine (for vegetarians) are available as dietary supplements [12, 14]. There are also in the literature studies on how the protective effect



(a) (b)

Figure 1. (a) Topaquinone, TPQ; (b) pyridoxal phosphate.

afforded by supplemental DAO from pea is modulated by different additives (such as cholic acids, bicarbonate, proteases, lipids, alcohol) [17, 18].

Quantitation of biogenic amines in foods is important for assuring the desired quality [19] but also because their concentration is a good biomarker for freshness, especially in fish and shellfish [20]. Besides the toxicity of histamine itself, it is important to mention that the presence of other biogenic amines has an enhancing effect on toxicity due to their inhibitory effect on histaminase activity [21]. Methods used to analyze biogenic amines are chromatography (HPLC [22], cation-exchange [23], GC [24], TLC [25]), fluorimetry [26], spectrophotometry [27], and chemoluminiscence [28]. These methods require expensive instrumentation, are time-consuming, some even requiring derivatization. Biosensors for biogenic amines have also been reported [29, 30] and they offer less costly and fast methods of analysis. However, they require high-activity, stable enzymes and mediators if amperometric detection (less expensive) is used.

While commercial DAO is expensive and has low activity (0.05 U/mg solid [31]), purification of the enzyme from natural sources (animal or vegetal) is labor-intensive and time-consuming [16, 32]. The present study uses a green approach to enhance and stabilize DAO from porcine kidney using high hydraulic pressure (HHP). HHP was used in industrial practice to control microbial growth thereby limiting biogenic amines formation in fermented foods. It replaced traditional methods like freezing and irradiation [11]. A mechanical procedure is presented herein in which DAO is subjected to HHP in the presence of enzyme-friendly redox species. DAO unfolds partially and subsequently refolds, after removal of HHP [33]. The fact that DAO-friendly redox units are entrapped upon refolding within the 3-D structure of the enzyme affords a self-mediated, "wired" DAO [34] with promise for use in biosensors for biogenic amines.

2. MATERIALS AND METHODS

2.1. Materials

Diamine oxidase from porcine kidney (EC 1.4.3.6) and peroxidase from horseradish (HRP, EC 1.11.1.7) were purchased, as lyophilized powders, from Millipore-Sigma. The other chemicals (pyridoxal phosphate, PLP, copper sulfate pentahydrate, CU, 3,5 dichloro-2-hydroxybenzene-sulfonic acid, DCHBS, 4-aminoantipyrine, AAP, and putrescine) were purchased from Millipore-Sigma, USA. They were reagent grade and used without further purification. Deionized water (18.2 MΩ cm^{-1}) was used in all experiments.

2.2. Methods

2.2.1. DAO assay

DAO activity was measured using an assay published in [35] and modified by us [4], in which the reaction catalyzed by DAO is coupled to a peroxidase reaction. Prior to the assay, a mixture (AAP-DCHBS-HRP) was prepared using 856 µL of 50 mM phosphate buffer pH 7.2, 19.5 µL of 100 mM AAP, 19.5 µL of 0.2 M DCHBS and 5 µL of HRP (7 mg/mL). A mixture of 400 µL of modified DAO/native enzyme solution (20 mg/mL) with 3 µL of putrescine was incubated for 20 min at room temperature. After incubation, 900 µL of the AAP-DCHBS-HRP was added to the sample mixture. 100 µL volumes of the above mixture were monitored at 515 nm, for 2 h, in a 96-well plate reader using 50 mM phosphate buffer pH 7.2 as blank. A colored compound was formed due to oxidative coupling of DCHBS with AAP and formation of a red quinoneimine species.

2.2.2. DAO transiently exposed to high hydraulic pressure

In two parallel experiments, DAO dissolved in 50 mM phosphate buffer pH 7.2 was compressed at 325 MPa for 30 min and for 60 min, respectively. Subsequently, four mixtures (DAO with different modifiers) were processed using the same protocol. The composition of the samples and the results are presented in Table 1 together with the conditions used for processing at high hydraulic pressure. The compression times shown reflect the duration each DAO sample was subjected to HHP, with or without a modifier present (at 2 mM, in 50 mM phosphate buffer pH 7.2). A Pressure Biosciences Inc. (PBI) High Pressure Generator was used to compress some samples, at 325 MPa for 60 min, while others were compressed at 325 MPa for 30 min. Each sample, after exposure to HHP, underwent dialysis for 24 h against 50 mM phosphate buffer pH 7.2.

Table 1. Modifier content and residual activities of DAO samples.

Enzyme sample	Compression Time	Entrapped Modifier %	DAO Activity[a] (nM/h)	Residual Activity (%)	"Wire" Effect[b] (%)
Native DAO	-	-	3.31	100	-
DAO60-ME	60 min	-	0.40	12.08	100
DAO60-PLP	60 min	18.78	0.76	22.96	190.00
DAO60-CU	60 min	47.05	3.83	115.71	957.50
DAO30-ME	30 min	-	0.76	22.96	100
DAO30-PLP	30 min	51.36	1.70	51.36	223.68
DAO30-CU	30 min	56.75	7.70	232.63	1013.16

[a]Activities of all modified DAO were adjusted for loss due to lyophilization (70%)
[b]Compared to DAO processed at the same compression time, without modifier

Subsequent lyophilization was done using a Labconco FreeZone 2.5 Freeze Dry System (at −55 °C and 0.05 mbar). This procedure is known to result in a decrease in activity of the enzyme, or even denaturation [33]. To account for this, a sample of the native DAO was assayed before and after lyophilization. The lyophilized samples were stored at −20 °C. The buffer from dialysis was saved to analyze its modifier content.

2.2.3. Analysis of the modifier entrapped in the modified enzymes (DAO-ME)

The modifier uptake for each modified enzyme (DAO-CU - processed in the presence of copper sulfate, and DAO-PLP – processed in the presence of PLP) was determined indirectly, using spectrophotometric measurements of each modifier species (Table 1) in the wash from the dialysis procedures (at 600 nm and ε = 942 $M^{-1}\cdot cm^{-1}$ for CU and at 388 nm and molar absorptivity ε = 5020 $M^{-1}\cdot cm^{-1}$ for PLP). The concentration of the modifier in the dialysis wash was calculated using Beer's Law, and the amount entrapped in the refolded enzyme ("wired" DAO) was calculated by subtraction from the amount used in the "wiring" (Table 1).

2.2.4. Fluorescence measurements

Fluorescence measurements were performed with a Horiba Fluoromax 4 spectrophotometer. The excitation wavelength was 280 nm, and the emission spectrum was monitored in the range 320-800 nm. All measurements were performed in 50 mM phosphate buffer pH 7.2, at 25 °C. The values on the ordinate ("Fluorescence"), as displayed in Figure 2 and Figure 3, are proportional to the emission of each sample.

2.2.5. Circular dichroism spectra

Circular dichroism (CD) spectra of the native DAO and of some of DAO-ME were obtained using a Jasco 815 Spectrophotometer, at 25 °C, using a 0.1 cm pathlength quartz cell. The concentrations were in the 0.07-0.15 mg/mL range and all samples were dissolved in 50 mM phosphate buffer pH 7.2. Ellipticity was measured between 190 and 300 nm at 1 nm intervals, corrected for buffer baseline and converted to molar ellipticity. Each CD spectrum was normalized per concentration, number of amino acids, and pathlength. Secondary structure assignments were obtained using the software CD PRO (Jasco, Inc).

2.2.6. DAO-ME biosensor

An enzyme electrode using the "wired" DAO with the highest activity (DAO-CU30, compressed for 30 min in the presence of $CuSO_4$ pentahydrate) as sensing element was built using a glassy carbon electrode (GCE, 3 mm diameter) from BASi Bioanalytical Systems, Inc. A 100 µL volume of 50 mM phosphate buffer pH 7.2 containing 1 mg of DAO30-CU was placed on the GCE and air-dried. Subsequently, a 20 µL volume of 0.75% glutaraldehyde (used as a cross-linker) was placed on top and air-dried. The biosensor was used as working electrode (WE) in a three-electrode cell,

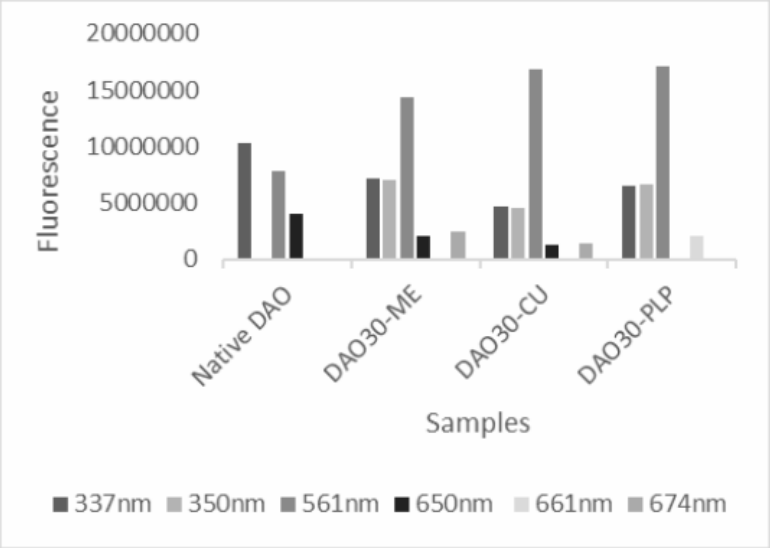


Figure 2. Fluorescence emission spectra of DAO samples.

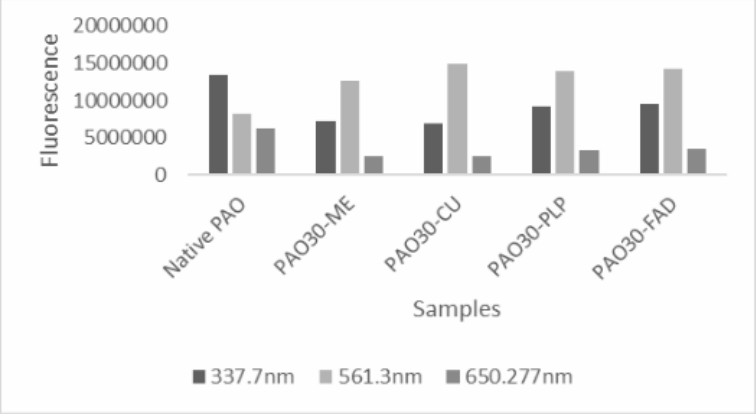


Figure 3. Fluorescence emission spectra of PAO samples.

with a standard calomel electrode (SCE) as reference, and a platinum (Pt) wire as counter electrode. The electrochemical cell contained 75 mL 50 mM phosphate buffer pH 7.2 and it was used in cyclic voltammetry experiments run using an Epsilon Electrochemical Station from BASi. To assess the catalytic effect of the "wired" DAO, cyclic voltammograms were generated in the following conditions: the cell containing only the phosphate buffer determined the background current, which was compared to the current measured after putrescine was added to the cell. Cyclic voltammetry was run between −0.2 V and +0.8 V, for 10 cycles, at a scan rate of 100 mV/s. The 10^{th} cycle was recorded and the i_{max} value was extracted from the voltammogram. The linearity of the biosensor response as a function of putrescine concentration was determined by taking cyclic voltammograms at different concentrations of putrescine.

3. RESULTS AND DISCUSSION

Although there are several animal and vegetal sources of DAO, free enzyme does not meet the demand of industrial applications due to limited stability and low activity [36, 37]. It is a known

fact that enzymes are more stable when they are immobilized and/or in the presence of their substrates. It has also been reported that oxidoreductases were enhanced by "wiring" [38, 39]. We reported the use of room-temperature ionic liquids (RTILs) as reversible denaturants to enhance oxidoreductases by using enzyme "wiring" [33, 34, 40, 41]. Therein, oxidoreductases (lactate dehydrogenase, cholesterol oxidase, plasma amine oxidase) were exposed to an RTIL in the presence of enzyme-friendly species intrinsic to the enzymes (coenzymes, metal ions). Following the removal of the RTIL (by dialysis), the enzymes refolded entrapping part of the modifiers present, which became "wires".

In this study, we present the results of molecular "wiring" of DAO achieved by using a reagentless procedure. DAO samples were transiently exposed to HHP with and without modifiers present. As modifiers, species relevant for DAO activity were chosen: divalent copper ions (cofactors, intrinsic to DAO) and pyridoxal phosphate. The latter was chosen due to its structural similarity to TPQ (DAO coenzyme) and lower cost. PLP is also the active form of vitamin B6 and, because it is recommended to heart patients, to Alzheimer's disease patients, it is beneficial in diabetes, in PMS, and in morning sickness, it was considered worth investigating potential synergistic effects in DAO modified using PLP. Upon compression at 325 MPa, the 3-D structure of the enzyme unfolds partially and subsequently refolds (after removal of HHP and dialysis). When HHP was applied with a modifier present, modifier molecules remained entrapped in the 3-D structure of DAO upon refolding. This fact does not make the entire insulating protein layer conductive. Still, it establishes preferential routes for electron transfer between the surface of the protein and prosthetic groups inside the enzyme (which would not be otherwise accessible for electron transfer which is a tunneling effect [42]). Thereby, these modifier units become molecular "wires" for diamine oxidase.

Several DAO samples were denatured/renatured using HHP, with or without redox modifiers present. Table 1 displays the residual activities following exposure to HHP and subsequent dialysis. The same table displays the modifier uptakes in the "wiring" procedures and their impact on enzyme activity ("Wire" effect). The DAO activity values (fourth column), which are compared to the native DAO, have been adjusted to reflect the loss in activity due to lyophilization. While in the literature it is stated that a loss of ~15–20% in amine oxidase activity was recorded following lyophilization [33], our tests on DAO resulted in a higher activity loss (~70%). DAO transiently exposed to HHP and renatured (without modifiers present, DAO-ME) retained partial activity compared to the native DAO. The residual activity was higher for DAO compressed for 30 min (DAO30-ME, 22.96%) compared to exposure for 60 min (DAO60-ME, 12.08%). These residual activities were substantially lower than those obtained for another copper amine oxidase (plasma amine oxidase, PAO) "wired" in our laboratory [33]. However, the trend is the same: shorter compression time is more beneficial (107.40% residual activity of PAO, at 30 min compared to 56.87% for 60 min exposure to HHP). Two published studies report the same influence of compression time on proteins. Compression times of 2-60 min were reported to affect the protein reversibly, while exposure beyond 60 min degraded the proteins [43, 44].

When RTILs were used as reversible denaturants for PAO, its residual activity was 87.80%, lower than the modification afforded by HHP exposure [33]. These differences between DAO and PAO (both homodimeric structures, with the same cofactor and coenzyme) after denaturation/ renaturation may be related, as suggested from inhibition studies [45] to an aspartic acid residue not found in other amine oxidases but found in DAO. It is positioned halfway down the negatively charged substrate channel and is responsible for DAO specificity since it interacts with the second amine group of diamines. In the case of lactate dehydrogenase (LDH), a flavin oxidoreductase with the coenzyme non-covalently retained in the 3D-structure of the protein, the residual activity after denaturation/renaturation using RTILs, without any modifier present, was higher (compared to native LDH) by factors between 2.1-4.8, depending on the enzyme:denaturant ratio [41]. In the case of

PAO, the different impacts of the two different procedures, one using HHP the other using RTILs as reversible denaturants, on residual activity, may be due to the fact that, while HHP at 325 MPa affects mainly hydrophobic interactions and much less H-bonds and ionic interactions within the 3-D structure of the protein, RTILs have a larger impact on secondary valence forces and on solvation as well.

In the molecular "wiring" procedures presented herein, two modifier species were tested, PLP and copper sulfate pentahydrate (CU). The modifier amounts retained within the renatured ("wired") DAO (DAO-PLP and DAO-CU, respectively) are shown in Table 1. The values are higher for DAO-CU (47.05 % for DAO60-CU and 56.75% for DAO30-CU), Cu^{2+} being an intrinsic component of DAO structure, compared to DAO-PLP (18.78% for DAO60-PLP and 51.36% for DAO30-PLP). The modified DAO, after refolding, while likely to adopt a slightly modified structure compared to native DAO, is also likely to have a 3-D structure close to the latter. The entrapment of PLP, somehow lower compared to that of copper ions, followed the same trend for PAO [33] with the difference that the values for 60 min/30 min are much closer, practically similar for PLP and almost identical with that for DAO60-PLP. While the trend is the same in DAO and PAO, the intake of $CuSO_4$ is higher in the latter [33]. We correlate this with the proximity (less than 15 Å) of a negatively charged residue (D445) to TPQ in each monomeric unit of PAO, making this region one of the most negative regions, able to attract positively charged units (in this case Cu^{2+}).

The "wiring" procedure was successful in enhancing DAO. Both modifiers used afforded enhancement ("wire" effect) compared to DAO-ME. For PLP, the activities ("wire" effects) were 190.00% for DAO60-PLP and 223.68% for DAO30-PLP, while for DAO60-CU the "wire" effect resulted in 957.50% activity and a value of 1013.68% for DAO30-CU (Table 1). Looking at these values, it becomes clear that divalent copper ions provide higher enhancement to DAO compared to PLP. The fact that copper ions are needed (together with oxygen) in the formation of TPQ (self-processing) as well [46] is yet another possible explanation for the activities displayed in Table 1. Low catalytic activities were reported in a study in which copper was replaced by other divalent cations (Ni^{2+}, Co^{2+}) much less efficient in the oxidation half reaction in which Cu^{2+}, intrinsic to DAO, catalyzes the electron transfer between reduced TPQ and oxygen [47].

It was reported in the literature that DAO from porcine kidney is inhibited by "excess" copper ions [48]. The entrapped amounts reported herein (Table 1), obviously, do not reach the required level for inhibition (300 mmol Cu^{2+}/100 mg DAO). "Wire" effects afforded to PAO by PLP and CU, respectively, showed higher enhancements for both modifiers compared to DAO but the same trend (higher for CU compared to PLP). The same was the case for PAO "wired" using RTILs [33]. Our method to introduce a molecular "wire" in an oxidoreductase was successfully applied to flavin oxidoreductases and reported for cholesterol oxidase [40] and for lactate dehydrogenase [41]. While flavin adenine dinucleotide (FAD) was used as a modifier in both cases (and was intrinsic to both enzymes), LDH showed the highest enhancement in activity (by a factor of 20+ for LDH) which corroborated the different binding of FAD inside the 3-D structure of the enzyme (non-covalent for LDH and both covalent and non-covalent in cholesterol oxidase).

The modified histaminase samples were stable. The activities of the "wired" DAO samples tested after 9 months (stored at -20 °C) showed residual values between 85-87% of those displayed after modification.

Fluorescence spectra were taken of the native DAO and of DAO modified using HHP under different conditions. Upon excitation at 280 nm, native DAO showed three emission bands at 337, 561, and 650 nm, respectively. The first band (337 nm) is due to tryptophan residues present within the structure of the protein [49]. After the enzyme was exposed to compression/denaturation and renaturation, the tryptophan band splits between 339 and 350 nm (Figure 2) and an additional emission band appeared at 674 nm. When a Trp residue is in a non-polar environment, an emission

maximum at 330 nm is expected. Red shifts are typically correlated with increase in polarity of the environment due to changes in interactions with water or charged residues following rearrangements or conformational modifications [50]. Water molecules close to the fused rings of Trp do not impact enough to result in a measurable red shift but, in conjunction with charged or dipolar groups (copper sulfate pentahydrate for instance), may result in a measurable shift whose magnitude depends on the variation in electrical fields along the axes of the indole unit of Trp. In the case of DAO-ME it appears that the enzyme has two kinds of tryptophan residues, less polar (339 nm) and more polar (350 nm). We attribute the bands appearing at 648 and 674 nm in the modified enzyme to a split of the band at 650 nm. This occurrence might be due to changes in TPQ (present in the active sites) interactions with the rest of the protein upon refolding. These interactions which are non-covalent, mainly van der Waals and H-bonding, may change as a result of molecular rearrangements and changes in electrical fields around TPQ units [51].

Following compression of DAO at 325 MPa in the presence of copper II ions, enzyme activity increased tenfold compared to that of DAO compressed without copper II ions present which was expected (Cu^{2+} being a cofactor for DAO). This enhancement was not dependent on the compression time. The increase in intensity of the emission band at 561 nm in the fluorescence spectra, compared to the corresponding band shown by DAO compressed without copper present, supports the activity data. However, fluorescence bands at longer wavelengths (648 and 672 nm), while being retained, were less intense. Red shift accompanied by decrease in fluorescence intensity was interpreted in another study [52] as indicative of protein denaturation due to Trp rearrangements and changes in tertiary structure of proteins. Our residual activity values, while accepting the occurrence of rearrangements leading to DAO with a modified tertiary structure, strongly eliminate denaturation as a result of the procedure proposed herein. The presence of PLP during compression/denaturation was beneficial, like that of copper, but afforded only a two-fold increase in the activity of the renatured DAO ("wire" effect). This effect was also independent of the compression time. The fluorescence spectrum for DAO30-ME showed suppression of the 650 and 674 nm bands and emergence of a new band in the middle (661 nm). This indicates that all three bands (650, 661 and 674 nm) may be related to specific chemical forms and/or conformations of PLP (closely related to TPQ, the prosthetic group) and its interactions with surrounding protein/solvating water molecules.

In the case of another dimeric copper enzyme, plasma mine oxidase (PAO), processed as above (30 min compression time), the fluorescence emission spectrum showed no additional emission bands (Figure 3). All three native bands (338, 561 and 650 nm) were retained after compression/ renaturation. That was true even for PAO modified in the presence of PLP (PAO30-PLP), copper sulfate (PAO30-CU), even FAD, a redox molecule without chemical/structural relationship to TPQ (PAO30-FAD). Like in the case of DAO, an increase in the emission band at 561 nm was recorded for PAO with the highest effect for the enzyme processes in the presence of copper II ions. The enhancements afforded by the denaturation/ renaturation using HHP resulted in high residual activities for PAO, as well [33].

Circular dichroism is an informative technique used to monitor conformational changes in proteins as a result of processing [53]. At present, the magnitude of such changes can be quantitatively evaluated using software packages. Some proteins display interconversions between different types of secondary structures which can be correlated to the protein function [54]. CD spectra were taken on the native DAO and on some of the modified samples in order to assess the impact of the "wiring" procedure on the secondary structure of DAO. The results are shown in Table 2. Far UV CD spectra (190-240 nm) give information about the transitions from α helix to β sheet structures, and about major changes in the backbone of the protein [55]. While denaturation/renaturation in the presence of copper sulfate leads to increase (4.7%) in helical content and decrease in β sheet presence (by 5.8%) following 60 min compression, it impacted oppositely when HHP was applied for 30 min only (7% decrease in helices with a more pronounced increase in β sheet content, 4.5%,

Table 2. Secondary structure assignments based on analysis of CD spectra performed with software CD PRO (Jasco, Inc.).

Sample	Assigned structure from CD scan (%) a helix/ b sheet/ b turn
Native DAO	24.8 / 24.7 / 21.8
DAO60-CU	29.6 / 18.9 / 21.7
DAO30-CU	17.8 /29.2 / 22.1
DAO60-PLP	18.7 / 30.0 / 21.0

resulting in the most active DAO-ME). The presence of PLP during processing showed the same trend: decrease in α helices content (6.1%) and increase in β sheet content (5.3%) without affording the same increase in activity as DAO30-CU. The β turn content remained unaffected by the "wiring" in all cases.

Detection and quantitation of biogenic amines is very important for clinical testing and food quality control. Since classical methods require expensive instrumentation, biosensing is a very desirable alternative. Amperometric biosensors, which combine the specificity of biological recognition with the sensitivity and low cost of amperometric transduction, are very good alternatives for diamine detection in complex matrices. Their shortcoming is the fact that they require mediators for the electron transfer between the prosthetic groups of the enzymes and electrodes to be efficient [56-59]. The "classical" approach to solve this problem was to immobilize enzymes together with mediators on electrodes. The procedure is labor-intensive when it involves covalent bonding [57], leads to decrease in enzyme activity, and is prone to mediator leakage [60, 61]. To address the electron tunneling requirement concerning the distance between redox centers [56], "wiring" of redox enzymes was introduced and tested on glucose oxidase, GOX [38, 39]. In this procedure, GOX was included in an osmium-based conductive gel, thereby reducing the distance for electron tunneling and resulting in faster kinetics. The approach seemed promising, and another group reported a layered composite made of alternating layers of GOX and osmium -derivatized poly(allylamine) layers, acting as redox relays [62]. In this setting the "wiring efficiency" was said to be controlled by the diffusion of electrons across layers. In a complex "wiring" procedure [63] the coenzyme FAD was separated from the holoenzyme, covalently bound to graphene nanoribbons, and then reattached to the apoenzyme (based on affinity).

While the "wiring" enzymes concept approached as above addresses the electron tunneling distance requirement, it does not result in direct electron transfer (DET) and it is not a reagentless approach to enzyme "wiring", as stated in [64]. This type of procedure affords mediated electron transfer (MET) through a "wire" which is external to the redox enzyme. In the present study, our approach to "wiring" is a molecular one. During compression, the native protein unfolds partially and, when modifiers are present, after removal of HHP and dialysis, it refolds trapping inside part of the modifier present and establishing thereby a preferential route for electron transfer. This route is shorter and functions as a molecular "wire" to redox centers inside DAO, which would not otherwise be accessible for electron transfer. Unlike the osmium or the ruthenium-based gels [65] reported in "wiring" of oxidoreductases, the modifiers used by us are non-toxic and enzyme-friendly.

We tested the most active molecularly "wired" DAO (DAO30-CU) in an amperometric biosensor, using putrescine as substrate. The enzyme electrode showed catalytic effect, measured as the difference between the current intensity in 5mM putrescine in buffer and the intensity of the current in the buffer only (values on lines 2 and 1 in Table 3 (*)). We consider this as another proof that the molecular "wiring" was successfully done using partial denaturation under HHP followed by renaturation after removal of HHP. The electrode was sensitive to putrescine without the presence of an external mediator. The DET was possible because redox centers buried deep in insulating native protein structure, and not involved in electron transfer with an electrode, following molecular "wiring" became participants in the electron transfer between DAO and electrode.

No quantitative relation can be established between the amount of modifier entrapped in the 3-D structure of modified DAO and the electron transfer mediating activity since "wires" effective as mediators are only those accessible to both prosthetic group of the enzyme and electrode (for

Table 3. Amperometric response of the DAO30-CU enzyme electrode.

Putrescine concentration (mM)	Current intensity (mA)
0	5.948*
5	7.173*
10	7.812
15	8.771
20	9.41
25	10.315
30	10.688
50	13.191

*Catalytic effect

electron tunneling to be feasible). This experiment was meant to prove that DAO modified using HHP shows promise for use in biosensors for the detection of biogenic amines. The lack of apparent correlation between the DAO-ME activity and the amperometric response could be attributed to the different impact of the modification under HHP on the two steps involved in the catalytic reaction, the oxidative and the reductive reactions, respectively [66]. The response was linear on a broad range of putrescine concentrations (0-50 mM) with a sensitivity of 0.1419 mA/mM. The response time of the DAO biosensor was 200 s. The operational stability of the biosensor was characterized by less than 7% loss in current intensity after repeated daily testing over 10 days (80 measurements), with storage in 50 mM phosphate buffer pH 7.2, at 4 °C, between tests. A biosensor for biogenic amines reported in the literature which used DAO and Prussian blue-indium tin oxide nanoparticles as mediator reported a sensitivity of 1.84 mA/mM [67]. Another biosensor reported previously and using DAO from porcine kidney and ferrocene derivatives as mediators displayed linearity up to 6 mM only [68]. Two other biosensors for histamine detection reported in [69, 70] had the same limitations (toxicity of the mediator and limited range of the linear response). For the DAO30-CU biosensor discussed herein, more concentrations need to be investigated, in the micromolar range, to determine the low detection limit and to focus on the intended applications in clinical laboratory and in assessing seafood freshness.

4. CONCLUSION

This study presents the successful molecular "wiring" of DAO under the action of HHP. DAO used in an amperometric biosensor needs, like any other oxidoreductase, a mediator for efficient electron transfer. Redox molecules used typically as mediators are toxic and require immobilization. The procedure presented herein, purely mechanical and reagentless, affords a modified/ self-mediated DAO able to perform DET. Avoiding use of toxic species, the procedure is environment-friendly as well. The fact that it is not labor-intensive makes it even more attractive and decreases the number of steps which could negatively impact DAO activity. The strategy to protect enzyme activity is present in the choice of modifiers as well. Divalent copper ions intrinsic to DAO and PLP, closely related to TPQ – prosthetic group of DAO, are non-toxic redox species closely related to the enzyme which makes this "wiring" procedure an enzyme-friendly one as well. Additional copper ions as "wire" were the most beneficial. The refolded DAO structures, although non-native ones, were stable ones. We correlate the enhancement afforded by the mechanical, reagentless procedure used herein to the successful entrapment of additional redox species which resulted in faster kinetics of the electron transfer, and hence enhanced DAO. The biosensor using self-mediated DAO30-CU as sensing element showed good promise, as proof of concept, for use in biogenic amines detection.

ACKNOWLEDGEMENTS

The authors thank Dr. Catherine Royer from Rensselaer Polytechnic Institute for generously allowing access to the High Pressure Generator used in the HHP modifications. The authors also thank Dr. Ken Yamaguchi from New Jersey City University for the help with the fluorescence spectra and for the useful comments. Dr. Pompilia Ispas-Szabo from L'Universite du Quebec a Montreal is gratefully acknowledged for the valuable help with the enzyme assays.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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