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Amine oxidase-based flow biosensor for the assessment of fish freshness

Ivo Frébort *, Lenka Skoupá, Pavel Peč

Department of Biochemistry, Faculty of Science, Palacký University, Šlechtitelů 11, 783 71 Olomouc, Czech Republic

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Abstract

Amine oxidases (EC 1.4.3.6) from grass pea (*Lathyrus sativus*) seedlings and fungus *Aspergillus niger* were immobilized to construct flow enzyme reactors for amine assay with spectrophotometric detection of enzymatically produced hydrogen peroxide by a peroxidase/guaiacol system. While immobilized amine oxidase from *A. niger* showed poor storage stability, the *L. sativus* enzyme-based system was found useful for assay of putrefactive amines (putrescine and histamine) as markers of fish meat decomposition. The optimized biosensor with average lifetime 20 days showed a linear response to the amount of histamine in the range 7.0–90 nmol with the assay limit of 4.4 nmol and putrescine in the range 0.9–70 nmol with the assay limit of 0.5 nmol.

Keywords: Amine oxidase; Freshness control; Biosensor

1. Introduction

Assay of amines in meat extracts especially from fish can be used as a freshness control, since the amines are produced in the meat putrefaction process. Histamine assay in fish meat has been widely conducted using a fluorometric method with *o*-phthalaldehyde (Staruszkiewicz, Waldron & Bank, 1977), however, the method requires an extensive pretreatment of the sample, being rather time and labor consuming. The cost can be reduced by application of a specific enzyme, copper-containing amine oxidase (EC 1.4.3.6) [amine: O_2 oxidoreductase (deaminating)] that catalyzes the oxidative deamination of biogenic amines to the corresponding aldehydes and ammonia accompanied by a two-electron reduction of molecular oxygen to hydrogen peroxide (McIntire & Hartmann, 1992):

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + NH_3 + H_2O_2.$$

Enzymatic method using a free-state enzyme, such as measuring the oxygen uptake by an oxygen electrode and *Aspergillus niger* AO–I (Ohashi et al., 1994), which is a recent application of the classical amine oxidase activity assay (Macholán, 1968), is relatively simple, but requires large quantities of purified enzyme. Amperometric biosensor based on the detection of oxygen uptake or hydrogen peroxide release with pea seedling (Toul & Macholán, 1975; Macholán & Slanina, 1991) or pig kidney amine oxidase (Male, Bouvrette, Luong & Gibbs, 1996; Bouvrette, Male, Luong & Gibbs, 1997) immobilized on a nylon net via glutaraldehyde, and bovine serum and *A. niger* amine oxidases immobilized on a collagen membrane (Karube, Satoh, Araki & Suzuki, 1980) are more advantageous. However, these methods require relatively large volume of the reaction mixture that can accommodate the electrode. Also an equilibration of the electrode with the reaction mixture before each assay is necessary. Recently, very advanced flow biosensor with immobilized pig kidney amine oxidase based on a chemiluminescent detection of hydrogen peroxide has been described (Alam, Sasaki, Watanabe & Maeyama, 1995). This biosensor overcomes most of previous problems, however requires special instrumentation that is not generally available. More applicable seems to be an amperometric biosensor based on a carbon paste with immobilized pea seedling amine oxidase and peroxidase that could be eventually applied as a postcolumn detector (Wimmerová & Macholán, 1996). A simple flow enzyme reactor based on the spectrophotometric detection of hydrogen peroxide is described in this study.

* Corresponding author. Tel.: +420-68-524-1198; fax: +420-68-522-1332.

E-mail address: frebort@prfholnt.upol.cz (I. Frebort)

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2. Material and methods

2.1. Enzymes, activity and protein assay

Amine oxidases from etiolated grass pea (*Lathyrus sativus*, cv. 'Newbred Troubsko') seedlings (Šebela et al., 1998) and the fungus *Aspergillus niger* AKU 3302 (Frébort et al., 1996) were purified as described previously and lyophilized. Specific activities of the enzymes assayed by the guaiacol method (Frébort, Haviger, & Peč, 1989) in 0.1 M potassium phosphate buffer, pH 7.0, were 1280 and 48 nkat/mg for the *L. sativus* amine oxidase and 8 and 54 nkat/mg for *A. niger* amine oxidase with substrates putrescine and histamine, respectively. Lyophilized horseradish peroxidase (100 U/mg) was obtained from Fluka (Buchs, Switzerland). Protein concentration was assayed according to Bradford, 1976.

2.2. Immobilization of the enzymes, determination of the coupling yield

HiTrap™ NHS-activated minicolumns (1.0 ml; Pharmacia, Uppsala, Sweden) were used for the immobilization. This columns contain Sepharose PH-34 micro activated with N-hydroxysuccinimide active ester (10 μmol/ml) on a nine carbon atom spacer. The column was prewashed with 6 ml ice cold 1 mM HCl using a peristaltic pump at the flow rate 1 ml/min and then the ligand solution was injected. Composition of the ligand solutions was as follows: (a) *System I* – 9 mg of *A. niger* amine oxidase and 1 mg of peroxidase in 1 ml of 0.1 M potassium phosphate buffer, pH 8.0, (b) *System II* – 3 mg of *L. sativus* amine oxidase and 0.5 mg of peroxidase in 1 ml of 0.1 M potassium phosphate buffer, pH 8.0, and (c) *System III* – 3 mg of *L. sativus* amine oxidase and 0.5 mg of peroxidase in 0.5 ml 20 mM potassium phosphate buffer, pH 7.0, and subsequently 0.5 ml of the same buffer without enzyme. The coupling was carried out at 4°C for 4 h. After the coupling, remaining ligand solution was eluted from the column with 3 ml of 0.1 M potassium phosphate buffer, pH 8.0, and mixed with 3 ml of 2 M glycine–HCl buffer, pH 2.0. The coupling yield was determined from the ratio of A_{280} before and after the coupling using the formula:

$$\text{Coupling yield}(\%) = 100 - [(A_{280}(2)(V_2 + V_3))/(V_1.A_{280}(1)].100$$

where $A_{280}(1)$ and $A_{280}(2)$ are absorbances at 280 of the ligand solution before and after the coupling, V_1 is the initial volume of the ligand solution, V_2 the volume used to elute remaining ligand solution out of the column and V_3 the volume of 2 M glycine-HCl buffer added.

Deactivation of remaining active groups was performed by repeated washing with buffers of pH 8.3 and 4.0 at room temperature. The washing was done at a flow rate of 1 ml/min. The column was washed with 6 ml of 0.5 M ethanolamine buffer containing 0.5 M NaCl, pH 8.3, then with 6 ml of 0.1 M acetate buffer containing 0.5 M NaCl, pH 4.0, and then again with 6 ml of 0.5 M ethanolamine buffer containing 0.5 M NaCl, pH 8.3. After that the column was left standing for 30 min. Then the column was washed with 6 ml of 0.1 M acetate buffer containing 0.5 M NaCl, pH 4.0, 6 ml of 0.5 M ethanolamine buffer containing 0.5 M NaCl, pH 8.3, and finally with 6 ml of 0.1 M acetate buffer containing 0.5 M NaCl, pH 4.0. After that the column was washed with 6 ml of running buffer, 0.1 M potassium phosphate buffer, pH 7.5, and used for amine assay.

2.3. Amine assay by the biosensor

The column with immobilized enzymes was connected to a System Gold liquid chromatograph equipped with a pump 125 NM and a diode array detector 168 NM (Beckman, Fullerton, CA, USA). The column was equilibrated with the reaction buffer, 20 mM potassium phosphate buffer, pH 7.0, as a mobile phase at the flow rate of 0.6 ml/min for 20 min at 25°C. Injected amine sample of total volume of 0.1 ml contained 0.05 ml of 0.035 M guaiacol and 0.05 ml of the amine containing sample (1–100 nmol). The response was monitored as a peak at 470 nm with the retention time of 2.5–3.9 min (varying with the amine concentration). Area of the peak reflected the amine concentration in the sample.

2.4. Determination of K_m values for free and immobilized enzyme

Michaelis constants for free amine oxidase with putrescine and histamine were calculated from initial rates of hydrogen peroxide production with variable substrate concentration determined by the guaiacol method (Frébort et al., 1989). The constants for the enzyme immobilized on the HiTrap minicolumn were calculated from initial rates of the response to variable substrate concentration at 470 nm defined as $v_0 = h/(t_R - t_D)$, where h is the peak height, t_R is the retention time of the peak and t_D the dead time of the column. In both cases the constants were calculated by nonlinear regression using the program Grafit 3.0 (Erithacus Software Ltd.) obtained from Sigma (St. Louis, MO, USA).

2.5. Preparation of fish meat extracts

Meat of rainbow trout (15 g portions) obtained from local market was left on air at 22°C for 1–15 days. The meat samples were homogenized by Ultra Turrax blender with 15 ml of 10% trichloroacetic acid used for deproteination and 1 ml of 1 mM sodium azide used as antibacterial agent and centrifuged at 6000 g for 15 min. Supernatant was then removed of lipids by extraction to

chloroform (15 ml). The chloroform layer was separated by centrifugation at 3500 g for 10 min. Upper aqueous layer was taken up and its pH was adjusted to 8.0 with potassium hydroxide prior to analysis or storage. If needed, the extracts were stored frozen at −30°C.

2.6. Amine assay by ninhydrin method

The reference assay of amines by ninhydrin method was carried out essentially as described earlier by Vejdělek and Kakáč (1969). Sample (1 ml) was mixed with 0.5 ml of the reagent (3% of ninhydrin in 0.2 M acetate buffer, pH 5.3, containing 0.01 potassium cyanide) and incubated on a boiling water bath for 15 min. Then the sample was diluted with 5 ml of 50% 2-propanol and shaken thoroughly. After cooling down to the laboratory temperature, the sample absorbance was measured at 570 nm against amine free blank sample. If the absorbance value exceeded 0.8, it was necessary to dilute again with another 5 ml of 2-propanol before reading the absorbance. The method was calibrated for assay of histamine in the range of 3–150 μmol.

3. Results and discussion

Amine level is a good indicator of putrefactive processes and its assay is used widely in freshness control (Staruszkiewicz et al., 1977). In this work, a novel approach was taken to construct a flow enzyme reactor based on the copper/quinoprotein amine oxidase that can be used with common liquid chromatography system. Protein coupling via N-terminus by displacement of N-hydroxysuccinimide to a HiTrap NHS-activated column was used to co-immobilize the amine oxidase and horseradish peroxidase. The matrix of the HiTrap column is fabricated from a Sepharose 4 Fast Flow covered with N-hydroxysuccinimide active ester on a nine-carbon spacer. Amine assay was then performed as a detection of the oxidation product hydrogen peroxide, liberated by the amine oxidase reaction, by subsequent reaction with peroxidase and guaiacol (*o*-methoxyphenol), producing yellow–brownish tetraguaiacoloquinone that was measured spectrophotometrically, as in the activity assay for amine oxidases (Frébort et al., 1989).

Initially, the amine oxidase from the fungus *Aspergillus niger* was used, since it has been reported suitable for histamine assay in free form (Ohashi et al., 1994) and has been recently commercially produced by food processing company Kikkoman in Japan. Coupling efficiency for the ligand mixture used was about 80% (System I). The column was connected to the HPLC system equipped with a diode array detector and the histamine was assayed as a peak area at 420 nm. Under experimental condition used (see Section 2, System I) the system produced a peak with retention time around 2.5 min and showed a linear response to the histamine amount in the range 2–50 nmol, with the assay limit of 2 nmol and optimum sample volume 0.1–0.5 ml. Unfortunately the stability of the immobilized enzyme was very poor with an average lifetime only 1–2 days after which it lost the amine oxidase activity, while the peroxidase activity was detected in the column even after one year storage at 4°C. To prolong the system lifetime, several techniques, such as dilution of the ligand mixture with a neutral protein and separate coupling of amine oxidase and peroxidase on two different columns with serial connection were applied, but the lifetime did not improve significantly. This observation complies with former study on immobilization of bovine serum and *A. niger* amine oxidases on a collagen membrane (Karube et al., 1980), where the *A. niger* amine oxidase showed only about 2.4% recovery in activity after immobilization comparing to the 22.4% for bovine plasma amine oxidase.

Since the experiments with the *A. niger* amine oxidase were not successful, further work focused on plant enzymes that have been well studied in our laboratory with regards to their kinetics and structure (Šebela et al., 1997; Šebela et al., 1998). On the basis of the screenings among Fabaceae plants (Luhová et al., 1998), the amine oxidase from grass pea (*Lathyrus sativus*) was selected as the one easy-to-purify in suitable amount and with high specific activity.

Two successful procedures for the enzyme reactor construction based on the grass pea amine oxidase (System II and System III) are shown in Section 2. Biosensor response to standard solutions of histamine and putrescine was tested at 420 and 470 nm. Typical peak of the response and an absorption spectrum of the colored product detected are shown in the Fig. 1. Major characteristics of the biosensors constructed are shown in Table 1. System II gave a linear response to histamine in the range 7.0–90.0 nmol with the detection limit of 4.4 nmol and to putrescine in the range 0.9–70.0 nmol with the assay limit 0.5 nmol. System III gave a linear response to histamine in the range 1.4–5.3 μmol with the detection limit of 0.2 μmol and to putrescine in the range 0.9–70.0 nmol with the assay limit 4.4 nmol. Above the linear ranges mentioned, the curve of the response versus amine amount became concave as the immobilized enzyme followed the Michaelis–Menten saturation kinetics. Although its coupling efficiency of the immobilization was lower (15.4% in average) the System II showed much better characteristics in terms of response and sensitivity to histamine than the System III (33.6%). This was likely due to different immobilization procedure which led to oversaturation of the Hi-Trap column with the amine oxidase protein in case of the System III. Subsequently, this oversaturation probably caused a dramatic structural change of the amine oxidase molecule and a sterical hindrance in the active site that was

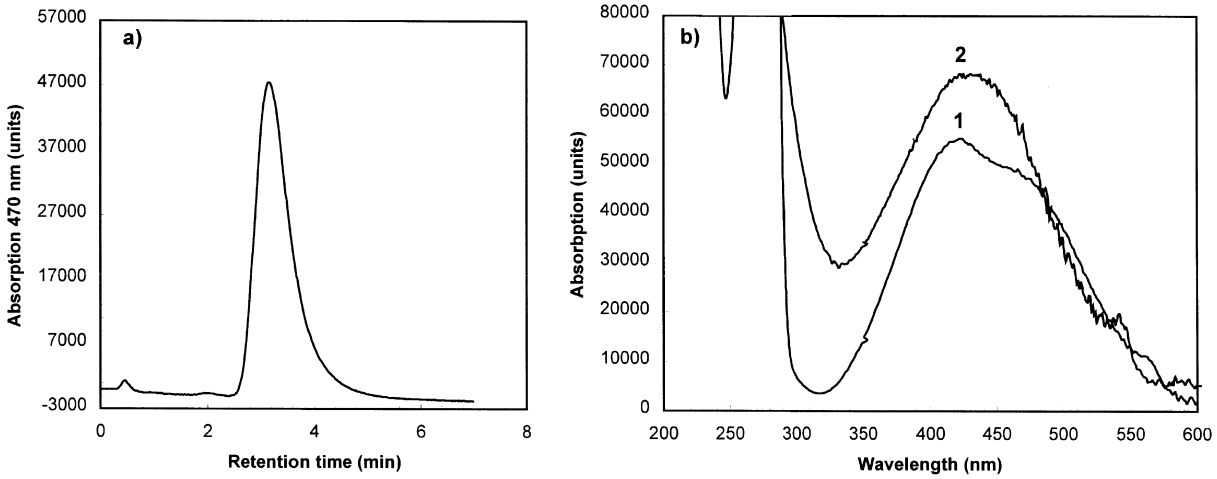

Fig. 1. Amine assay by the biosensor based on the grass pea amine oxidase (System II). (a) Typical peak of the response at 470 nm (20 nmol of histamine); (b) Spectrum of the colored product detected with the samples: (1) 20 nmol of histamine in standard solution, (2) fish meat extract containing the same amount of histamine. Refer to Section 2 for experimental details. The absorption is given in relative units as recorded by the diode array detector.

reflected also by two magnitude increase of the K_m for histamine in comparison with free enzyme (Table 1). Values of K_m for the System II did not differ much from those for free enzyme, which suggests that the immobilized enzyme retained its native characteristics.

Hence, the immobilization procedure used for the System II was then used thoroughly. Stability of this enzyme reactor is shown in Fig. 2(a). When the reactor was first stored at 4°C, the response dropped rapidly to approximately 60% of the original value and then remained constant for about 20 days after which slowly faded. The rapid drop in the response when first stored in cold is thought to be due to irreversible changes in the structure of the immobilized enzyme that eventually led to its stabilization. When measuring the samples of concentrated fish meat extracts, the assay at 420 nm was affected by remaining absorbance of the blank with maximum in the UV region which tail was reaching to about 450 nm (Fig. 1(b)). To avoid positive errors due to the blank absorption, the detection wavelength was shifted to 470 nm. The extinction coefficient for the guaiacol method at pH 7.0, $\varepsilon_{436} = 4.0$ mM^{-1} cm^{-1} was determined earlier (Frébort et al., 1989). From the absorption spectrum shown in Fig. 1, ε_{420} and ε_{470} were 4.1 and 3.7 mM^{-1} cm^{-1}, respectively, so shifting the detection wavelength from 420 to 470 nm decreased the sensitivity by only about 10%. The System II was calibrated for determination of putrescine up to 70 nmol and histamine up to 90 nmol as shown in the Fig. 2(b), with assay limits of 0.5 and 4.4 nmol, respectively. The assay limits were better than some of the amine assay already published such as 652 nmol of histamine for the oxygen sensor-based assay using free *A. niger* amine oxidase (Ohashi et al., 1994) or 259 nmol of putrescine and 304 nmol of histamine for the amperometric biosensor with pig kidney amine oxidase (Bouvrette et al., 1997). Although the detection limits reached in this work could not compare to those for chemilumine-

Table 1
Characteristics of the amine biosensors based on copper amine oxidase. Assay limits were determined as triplicated value of the signal over the noise

Immobilization (coupling efficiency)		K_m (mM)	Linear range (nmol)	Assay limit (nmol)	Average lifetime (days)
System I (*A. niger*)[a] (80.0%)	Histamine	n.d.	2.0–50.0	2.0	1–2
	Putrescine[b]	n.d.	n.d.	n.d.	
System II (grass pea)[a] (15.4%)	Putrescine	0.48	1.0–70.0	0.5	20
	Histamine	0.75	7.0–90.0	4.4	
System III (grass pea)[a] (33.6%)	Putrescine	0.55	7.0–90.0	4.4	20
	Histamine	16.3	1400–5300	200	
Free enzyme (grass pea)	Putrescine	0.29	–	–	–
	Histamine	0.35	–	–	

[a] See Section 2 for details of the immobilization procedure.
[b] Putrescine is a poor substrate for the *A. niger* amine oxidase, no data were obtained.

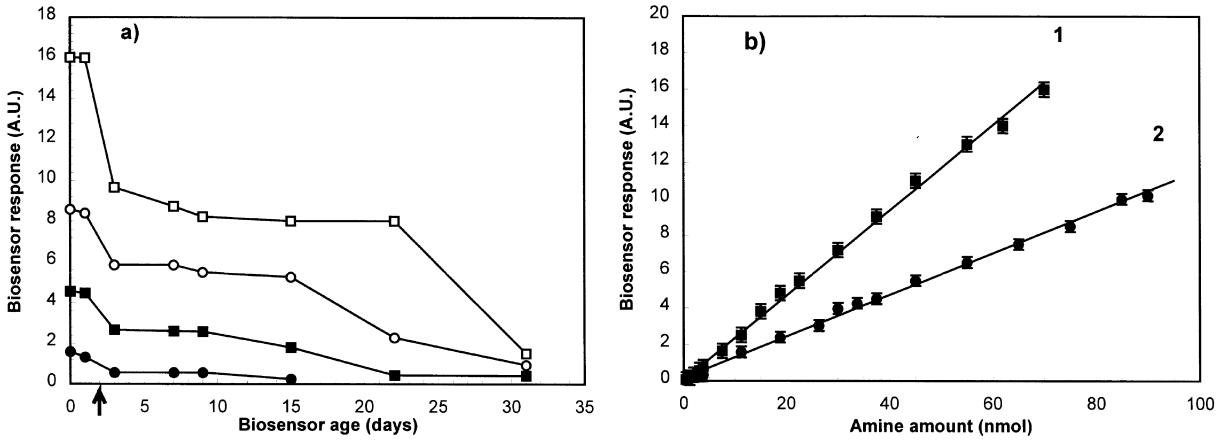

Fig. 2. Properties of the grass pea amine oxidase based biosensor (System II). (a) The response to selected putrescine amounts (● – 3.75, ■ – 11.25, ○ – 18.75, and □ – 37.5 nmol) during six week period; the arrow indicates the transfer of the enzyme reactor to the storage at 4°C. (b) Calibration curves for determinations of: (1) putrescine and (2) histamine (one week after the enzyme reactor preparation). The data are averages from three independent experiments.

scence-based postcolumn detectors (5–10 pmol) as described by Alam et al., 1995, the method presented has certain advantage over the chemiluminescence-based one, since it operates with common laboratory equipment and inexpensive chemicals.

Extracts of rainbow trout meat were prepared as described in Section 2. Increase in amine concentration during storage at 22°C, expressed as histamine amount per fish initial wet weight determined by the method presented is shown in Fig. 3. Amine extraction yield was determined by repeated extraction method on several different samples and was found to be about 80%. For comparison, a classical ninhydrin method for amine assay was used, which, however, gave in average 45% higher results due to nonspecific reaction with free aminoacids liberated by the meat decay. Fish samples that underwent one day storage at ambient temperature or repeated freezing and thawing showed elevated amine concentration and were clearly found by the enzyme reactor described, which was thus able to detect a spoiled fish meat before any odor appeared.

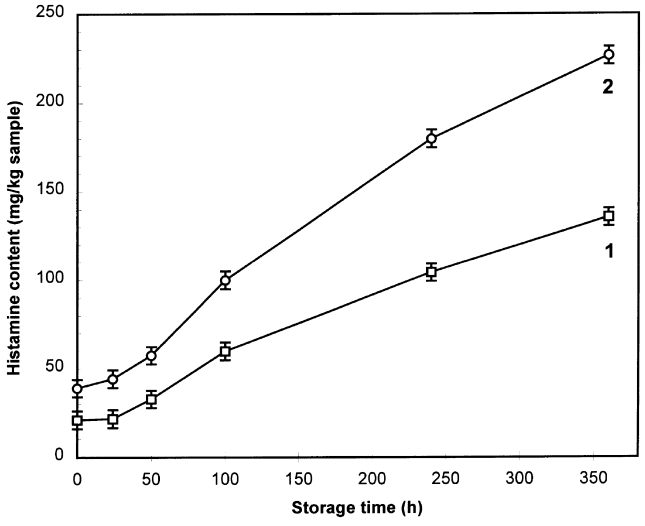

Fig. 3. Increase in histamine level in rainbow trout meat upon storage at room temperature as determined: (1) by the grass pea amine oxidase based biosensor (System II) and (2) the ninhydrin method as a reference. The data are averages from three independent experiments.

The experiments were focused on the assay of histamine as the most biologically active amine that affects normal functions of the heart, smooth muscles, motor neurons and gastric acid secretion. In most of European countries, the maximum levels allowed in the food are 100 mg of histamine per kg (Tombelli & Mascini, 1998). Histamine is also a critical freshness marker for dark flesh fish, i.e. scombroid fishes such as mackerel, tuna, saurel, etc. (Ohashi et al., 1994). In a white flesh fish (i.e. most of freshwater fishes, solefish, turbot), the critical freshness marker may be rather the level of diamines putrescine and cadaverine (Ohashi M., personal communication), that are recognized by our biosensor even more readily than histamine (assay ranges putrescine 0.5–70 nmol, histamine 4.4–90 nmol).

Acknowledgements

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