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Redox Hydrogel-Based Amperometric Bienzyme Electrodes for Fish Freshness Monitoring

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This work presents the design and optimization of amperometric biosensors for the determination of biogenic amines (e.g., histamine, putrescine, cadaverine, tyramine, cystamine, agmatine, spermidine), commonly present in food products, and their application for monitoring of freshness in fish samples. The biosensors were used as the working electrodes of a three-electrode electrochemical cell of wall-jet type, operated at −50 mV vs Ag/AgCl, in a flow injection system. Two different bienzyme electrode designs were considered, one based on the two enzymes [a newly isolated and purified amine oxidase (AO) and horseradish peroxidase (HRP)] simply adsorbed onto graphite electrodes, and one when they were cross-linked to an Os-based redox polymer. The redox hydrogel-based biosensors showed better biosensors characteristics, i.e., sensitivity of 0.194 A M^{-1} cm^{-2} for putrescine and 0.073 A M^{-1} cm^{-2} for histamine, and detection limits (calculated as three times the signal-to-noise ratio) of 0.17 μM for putrescine and 0.33 μM for histamine. The optimized redox hydrogel-based biosensors were evaluated in terms of stability and selectivity, and were used for the determination of total amine content in fish samples kept for 10 days in different conditions.

Rapid evaluation of fish and meat quality is permanently required in food industry, motivating a continuous search for freshness biomarkers and efforts to develop simple and inexpensive methods for their determination. Among these biomarkers, inositol monophosphate, hypoxanthine, and xanthine, which are intermediate degradation products of nucleic acids,[1,2] and some biogenic amines, such as histamine,[3−7] putrescine,[8,9] and cadaverine,[8,10] produced by microbial decarboxylation of the amino acids histidine, ornithine, and lysine, respectively, have been proposed. The biogenic amine content of various foodstuffs has been intensively studied due to their potential toxicity.[11] Histamine is the most biologically active compound from that class, affecting the normal functions of heart, smooth muscle, motor neurons, and gastric acid secretion.[12] Other biogenic amines, such as putrescine and cadaverine, may amplify the effects caused by histamine intoxication, inhibiting the enzymes involved in histamine biodegradation: diamine oxidase and histamine-*N*-methyl transferase.[13] Numerous countries adopted maximum levels for histamine in food, especially in fish products; e.g. Italian laws fixed this level at 100 mg kg^{-1} food,[3] and similar limits have been adopted by EEC regulations.[6]

Classical methods for the analysis of biogenic amines generally involve chromatographic techniques, such as gas chromatography,[14] thin-layer chromatography,[15] reversed-phase liquid chromatography,[15,16] and liquid-chromatography with derivatization techniques.[17−19] However, they often require sample pretreatment steps and skilled operators, and the relatively long analysis time and high costs make these methods unsuitable for routine use.

Enzymatic determination of biogenic amines was previously carried out and represents an alternative that can solve the above-mentioned problems. In this context, amperometric,[9−20] spectrophotometric,[21−24] fluorimetric,[25] or chemiluminometric detection methods[14,26] have been used. Amperometric electrodes using AO as the biological recognition element were also previously reported,

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(1) Volpe, G.; Mascini, M. *Talanta* **1996**, *43*, 283−289.
(2) Gruger, E. *J. Agric. Food Chem.* **1972**, *20*, 781−784.
(3) Tombelli, S.; Mascini, M. *Anal. Chim. Acta* **1998**, *358*, 277−284.
(4) Male, K. B.; Bouvrette, P.; Luong, J. H. T.; Gibbs, B. F. *J. Food Sci.* **1996**, *61*, 1012−1016.
(5) Bouvrette, P.; Male, K. B.; Luoung, J. H. T.; Gibbs, B. F. *Enzymol. Microb. Technol.* **1997**, *20*, 32−38.
(6) Chemnitius, G. C.; Bilitewski, U. *Sens. Actuators B 32*, **1996**, 107−113.
(7) Ohashi, M.; Nomura, F.; Suzuki, M.; Otsuka, M.; Adachi, O.; Arakawa, N. *J. Food. Sci.* **1994**, *59*, 519−522.
(8) Yano, Y.; Yokoyama, K.; Tamiya, E.; Karube, I. *Anal. Chim. Acta* **1996**, *320*, 269−276.
(9) Chemnitius, G. C.; Suzuki, M.; Isobe, K. *Anal. Chim. Acta* **1992**, *263*, 93−100.
(10) Gasparini, R.; Scarpa, M.; Di Paolo, M. L.; Stevanato, R.; Rigo, A. *Bioelectrochem. Bioenerg.* **1991**, *25*, 307−315.
(11) Taylor, S. L. *Crit. Rev. Toxicol.* **1986**, *17*, 91−128.
(12) Sttraton, J. E.; Hutkins, R. W.; Taylor, S. *J. Food Protect.* **1991**, *54*, 460−470.
(13) Hui, J. Y.; Taylor, S. L. *Toxicol. Appl. Pharmacol.* **1985**, *8*, 241−249.
(14) Bachrach, U.; Plesser, Y. M. *Anal. Biochem.* **1986**, *152*, 423−431.
(15) Yang, X.; Rechnitz, G. A. *Electroanalysis* **1995**, *7*, 105−108.
(16) Hauschild, M. Z. *J. Chromatogr.* **1993**, *630*, 397−401.
(17) Hui, J. Y.; Taylor, S. L. *J. AOAC Int.* **1983**, *66*, 853−857.
(18) Yen, G. C.; Hsieh, C. L. *J. Food Sci.* **1991**, *56*, 158−160.
(19) Veciana-Nogues, M. T.; Hernandez-Jover, T.; Marine-Font, A. *J. AOAC Int.* **1995**, *78*, 1045−1050.
(20) Xu, C. X.; Marzouk, S. A. M.; Cosofret, V. V.; Buck, R. P.; Neuman, M. R.; Sprinkle, R. H. *Talanta* **1997**, *44*, 1625−1632.
(21) Matsumoto, T.; Suzuki, O.; Katsumata, Y.; Oya, M.; Suzuki, T.; Nimura, Y.; Hattori, T. *J. Cancer Res. Clin. Oncol.* **1981**, *100*, 73−84.
(22) Stevanato, R.; Mondovi, B.; Sabatini, S.; Rigo, A. *Anal. Chim. Acta* **1990**, *273*, 391−397.
(23) Rinaldi, A. C.; Sanjust, E.; Rescigno, A.; Finazziagro, A.; Rinaldi, A. *Biochem. Mol. Biol. Int.* **1994**, *34*, 699−704.
(24) Toul, Z.; Macholán, L. *Collect. Czech. Chem. Commun.* **1975**, *40*, 2208−2217.
(25) Hungerford, J. M.; Arefyev, A. A. *Anal. Chim. Acta* **1992**, *261*, 351−359.

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both in single[10,27] and coupled enzyme-based designs.[15,28] However, most of the AO biosensors required a high applied potential (>500 mV vs Ag/AgCl),[5,27] which can lead to high background currents and bias signals caused by interferants present in complex matrixes to be analyzed.

Therefore, in this work, a bienzymatic approach based on a recently isolated and purified amine oxidase from grass pea (AO) and horseradish peroxidase (HRP) immobilized on solid graphite has been considered, focusing on electrode designs which operate at a low potential where biases from interferants are minimal. Bienzyme electrodes were prepared by simply adsorbing the two enzymes on the electrode surface using a direct electron-transfer approach and by applying the principle of a mediated electron-transfer cross-linking the enzymes and a redox polymer formed of poly(1-vinylimidazole) complexed with [Os(4,4′-dimethylbipyridine)$_2$Cl]$^{+/2+}$ (PVI$_{13}$-dmeOs) using poly(ethylene glycol) diglycidyl ether (PEGDGE) as the cross-linking agent. The optimal biosensor design was evaluated in terms of sensitivity, lifetime, and selectivity, and it was used for the analysis of fish samples stored under different conditions.

EXPERIMENTAL SECTION

Materials. Amine oxidase from grass pea (EC 1.4.3.6, AO) was isolated and purified according to a previously published protocol.[29] Peroxidase from horseradish (EC 1.11.1.7, HRP) was purchased from Sigma Chem. Co., St. Louis, MO (cat. no. P-6782) as a lyophilized powder with a declared activity of 1100 U mg^{-1} solid. Histamine dihydrochloride (cat. no. 100340), putrescine dihydrochloride (cat. no. 100450), tyramine hydrochloride (cat. no. 103173), cystamine dihydrochloride (cat. no. 100492), agmatine sulfate (cat. no. 100274), spermidine phosphate salt (cat. no. 102943) were from ICN Biochemicals Inc., Aurora, OH. Ethylenediamine (cat. no. 800947) was from Merck, Darmstadt, Germany. Cadaverine dihydrochloride (cat. no. C-8561) was purchased from Sigma. *Z*- And *E*-2-butene-1,4-diamino dihydrochloride were synthesized according to previously published protocols.[30,31] PVI$_{13}$-dmeOs was prepared by complexing poly(1-vinylimidazole) with [osmium(4,4′-dimethylbipyridine)$_2$Cl]$^{+/2+}$, as described elsewhere.[32] Poly(ethylene glycol) (400) diglycidyl ether (PEGDGE, Polysciences, Warrington, PA, cat. no. 08210) was used for cross-linking AO and HRP to the osmium-complexed polycation. Disodium hydrogen phosphate dihydrate and potassium dihydrogenphosphate, purchased from Merck (cat. no. 1.06580 and no. 1.04873, respectively), and water purified in a Milli-Q system (Millipore, Bedford, MA) were utilized to prepare the phosphate buffer 0.1 M, pH 7.2 (PB), used as the carrier solution and supporting electrolyte in the flow injection experiments, if not otherwise stated. Hydrogen peroxide, 35 wt % water solution was from Across Organics, Geel, Belgium.

(26) Alam, M. K.; Sasaki, M.; Watanabe, T.; Maeyama, K. *Anal. Biochem.* **1995**, *229*, 26−34.
(27) Draisci, R.; Volpe, G.; Lucentini, L.; Cecilia, A.; Frederico, R.; Palleschi, G. *Food Chem.* **1998**, *62*, 225−232.
(28) Gasparini, R.; Scarpa, M.; Vianello, F.; Mondovi, B.; Rigo, A. *Anal. Chim. Acta* **1994**, *294*, 299−304.
(29) Šebela, M.; Luhová, L.; Frébort, I.; Faulhammer, H. G.; Hirota, S.; Zajoncová, L.; Stužka, V.; Peč, P. *Phytochem. Anal.* **1998**, *9*, 211−222.
(30) Peč, P.; Chudý, J.; Macholán, L. *Biológia (Bratislava)* **1991**, *46*, 665−672.
(31) Macholán, L.; Hubálek, F.; Šubová, H. *Collect Czech. Chem. Commun.* **1975**, *40*, 1247−1265.
(32) Ohara, T. J.; Rajagopalan, R.; Heller, A. *Anal. Chem.* **1994**, *66*, 2451−2457.

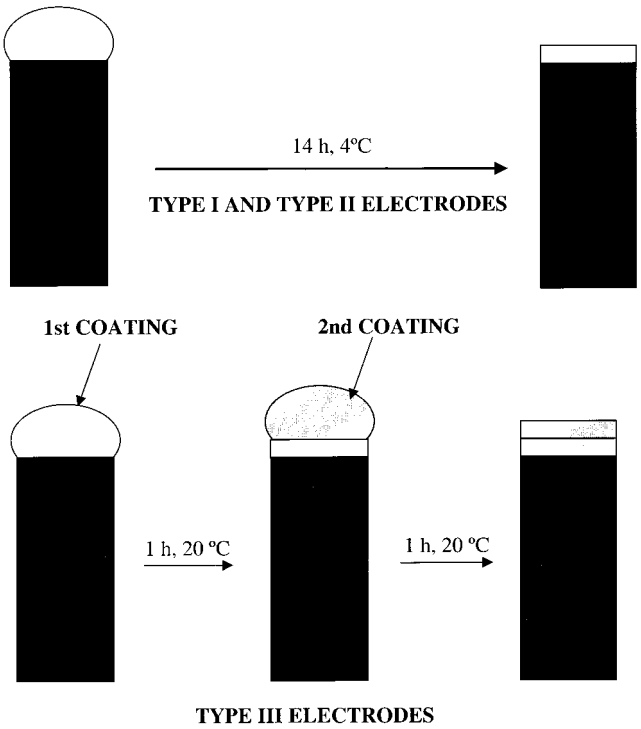

Figure 1. Preparation of AO−HRP bienzyme electrodes: type I electrodes, AO + HRP (mixture); type II electrodes, AO + HRP + PVI$_{13}$-dmeOs + PEGDGE (mixture); Type III electrodes, (a) first coating, HRP + PVI$_{13}$-dmeOs + PEGDGE (mixture); second coating, AO; (b) first coating, AO; second coating, HRP + PVI$_{13}$-dmeOs + PEGDGE (mixture); (c) first coating, HRP; second coating, AO + PVI$_{13}$-dmeOs + PEGDGE (mixture); (d) first coating, AO + PVI$_{13}$-dmeOs + PEGDGE (mixture); second coating, HRP.

All experiments were performed at room temperature. All solutions were daily prepared using PB as solvent, if not otherwise stated, and were filtered through 0.45 μm (Millipore, Molshem, France, type HA) filters and degassed before use.

Biosensor Preparation. Bienzyme graphite electrodes were prepared as follows: first, rods of spectroscopic graphite (Ringsdorff Werke GmbH., Bonn, Germany, type RW001, 3.05 mm diameter) were cut and polished on a wet fine emery paper (Tufback, Durite P1200, Allar, Sterling Heights, MI), followed by rinsing the electrode surface with water and drying at room temperature before coating with enzymes. Three different electrode types were prepared (see Figure 1):

Type I electrodes were prepared by placing 6 μL of a premixed solution containing various amounts of AO (stock 20 mg mL^{-1}) and HRP (stock 10 mg mL^{-1}) on the graphite electrode (direct electron-transfer approach).

Type II electrodes were prepared using 6 μL of a mixture formed of AO (same as above), HRP (same as above), PVI$_{13}$-dmeOs (stock 10 mg mL^{-1}), and PEGDGE (5 mg mL^{-1} water solution freshly prepared and used within 15 min) in different w/w (%) ratios placed on the top of the graphite electrode (one-layer electrodes).

Type III electrodes were prepared using a sequential coating procedure. The four components of the above-described mixture were separated in two groups; 6 μL of the first one were initially added on the graphite, and after drying, the second layer formed by 6 μL of the other group of components was placed over the first one (two layer electrodes), as follows:

Type IIIa. First, 6 μL of a premixed solution of HRP, PVI_{13}-dmeOs, and PEGDGE was placed on the top of the electrode. Next, the electrodes were dried for 1 h before coating with 6 μL of AO.

Type IIIb. First 6 μL of AO was placed on the top of the electrode. After drying for 1 h, 6 μL of a premixed solution of HRP, PVI_{13}-dmeOs, and PEGDGE was added.

Type IIIc. In the first step, 6 μL of HRP was placed on the top of the electrode, and after drying, a second layer containing 6 μL of a premixed solution of AO, PVI_{13}-dmeOs, and PEGDGE was added.

Type IIId. First, a premixed solution formed of 6 μL of AO, PVI_{13}-dmeOs, and PEGDGE was placed on the top of the electrode. Next, electrodes were dried for 1 h before coating with 6 μL of HRP.

The amount of all the components added on type III electrodes was constant and the same as the optimum found for the type II electrodes.

If not otherwise stated, all modified electrodes were stored at 4 °C for 14 h in a glass beaker and were rinsed with PB before use. All results presented in this paper are means of at least three equally prepared electrodes.

Instrumentation. The bienzyme-modified graphite electrodes were inserted as the working electrode in a single channel flow injection system[33] containing a manual injection valve (Valco Instruments Co. Inc., Houston, TX) with a 50 μL injection loop and a three-electrode flow-through wall-jet type electrochemical cell. A peristaltic pump (Alitea AB, Stockholm, Sweden) was used to pump the carrier solution at desired flow rates through Teflon tubings (0.5 mm i.d.) to the flow cell. A potentiostat (Zäta-Electronik, Höör, Sweden) maintained the constant potential between the working and the Ag/AgCl (0.1 M KCl) reference electrode. A platinum wire was used as the counter electrode. The response current was monitored with a single channel strip-chart recorder (Model BD 111, Kipp & Zonen, Delft, The Netherlands).

Operational stability experiments were made using an automated sample injection analyzer (Ismatec, Glattburg-Zürich, Switzerland) by injecting samples of 100 μM histamine and 50 μM putrescine, respectively, with a sample throughput of 30 injections h^{-1}, using PB as the carrier solution at a flow rate of 0.5 mL min^{-1}.

Sample Preparation. The frozen fish-muscle samples (turbot, *Psetta maxima*) were kindly provided by Dr. Gunilla Önning, Deparment of Applied Nutrition and Food Chemistry, Lund University, Sweden. Triplets of 1.0-g fish samples kept under different conditions were homogenized in 10 mL of PB. The homogenate was centrifuged at 13000*g* for 60 min. at 4 °C. The supernatant was separated and immediately analyzed by direct injection into the flow system.

RESULTS AND DISCUSSION

Amine oxidases represent a class of enzymes with a ubiquitous distribution in mammals, plants, and microorganisms.[34,35] However, the structure, selectivity, and biological functions are very different, depending on the isolation source. The grass pea amine oxidase,[29] used during this work, is a copper-containing AO, which besides the metal ions contains also an organic cofactor with a quinoide structure (topa quinone) in its catalytic site,[36] most of the proposed reaction mechanisms being related to the structure of this cofactor. However, in most of the developed methods for monitoring of biomarkers using an amine oxidase, the enzyme is converting the analyte to a corresponding aldehyde with NH_3 and H_2O_2 release, according to reaction 1:

$$RCH_2NH_2 + H_2O + O_2 \rightarrow RCHO + H_2O_2 + NH_3 \quad (1)$$

Both, the oxygen consumption[7,37] and hydrogen peroxide formation[3,4] have been used as detection principles of biogenic amines based on the above-mentioned reaction, usually requiring high overvoltages. We recently presented a biosensor design that is based on the same enzyme as the one used in this work[38] displaying very good characteristics but still requiring a quite high applied potential (+200 mV vs Ag/AgCl). This aspect represents a serious drawback when considering monitoring in complex matrixes (e.g., food samples).

The combination of peroxidases with hydrogen peroxide-producing oxidases for the development of amperometric biosensors has been extensively used during the past years.[39,40] The possibility of using biosensors based on coupled enzymes using a direct electron transfer between a peroxidase and an appropriate electrode at low applied potentials (around 0 mV vs SCE)[41] makes these types of biosensors theoretically more suitable for applications in real matrixes. AO also has been previously coupled to peroxidases,[15,28] but the obtained biosensors worked at high potential (+200 mV),[28] being prone for biases when used in complex matrixes. All these electrodes used, however, AOs from different sources and, hence, with different characteristics.

The bienzyme approaches outlined in this work were based on the direct coupling of AO and HRP either simply immobilized on the electrode surface (type I) or cross-linked into a redox hydrogel (types II and III). In all configurations, the amine oxidase first converts the amine substrate (e.g., histamine) to an aldehyde product, the active form of the enzyme being recovered by oxidation of the organic cofactor in the presence of molecular oxygen (see Figure 2). The hydrogen peroxide formed during the first reaction is subsequently reduced to water by peroxidase; its native form being regenerated either by direct reduction of its heme cofactor on the electrode surface (see Figure 2a, type I biosensors) or by receiving electrons from a mediator, maintained in its reduced form by the potential applied on the graphite electrode (see Figure 2b, type II biosensors).

(33) Appelqvist, R.; Marko-Varga, G.; Gorton, L.; Torstensson, A.; Johansson, G. *Anal. Chim. Acta* **1985**, *169*, 237−242.

(34) Rinaldi, A.; Floris, G.; Giartosio, A. *Structure and functions of amine oxidases*; CRC Press: Boca Raton, FL, **1985**.

(35) Knowles, P. F.; Dooley, D. M. *Metal ions in biological systems*; Marcel Dekker: New York, 1994.

(36) Janes, S. M.; Mu, D.; Wemmer, D.; Smith, A. J.; Kaur, S.; Maltby, D.; Burlingame, A. L.; Klinman, J. P. *Science* **1990**, *248*, 981−984.

(37) Karube, I.; Satoh, I.; Araki, Y.; Suzuki, S. *Enzymol. Microb. Technol.* **1980**, *2*, 117−120.

(38) Niculescu, M.; Frébort, I.; Peč, P.; Galuszka, P.; Mattiasson, B.; Csöregi, E. In press.

(39) Vijayakumar, A. R.; Csöregi, E.; Heller, A.; Gorton, L. *Anal. Chim. Acta* **1996**, *327*, 223−234.

(40) Ghobadi, S.; Csöregi, E.; Marko-Varga, G.; Gorton, L. *Curr. Separations* **1996**, *14*, 94−102.

(41) Marko-Varga, G.; Emnéus, J.; Gorton, L.; Ruzgas, T. *Trends Anal. Chem.* **1995**, *14*, 319−328.

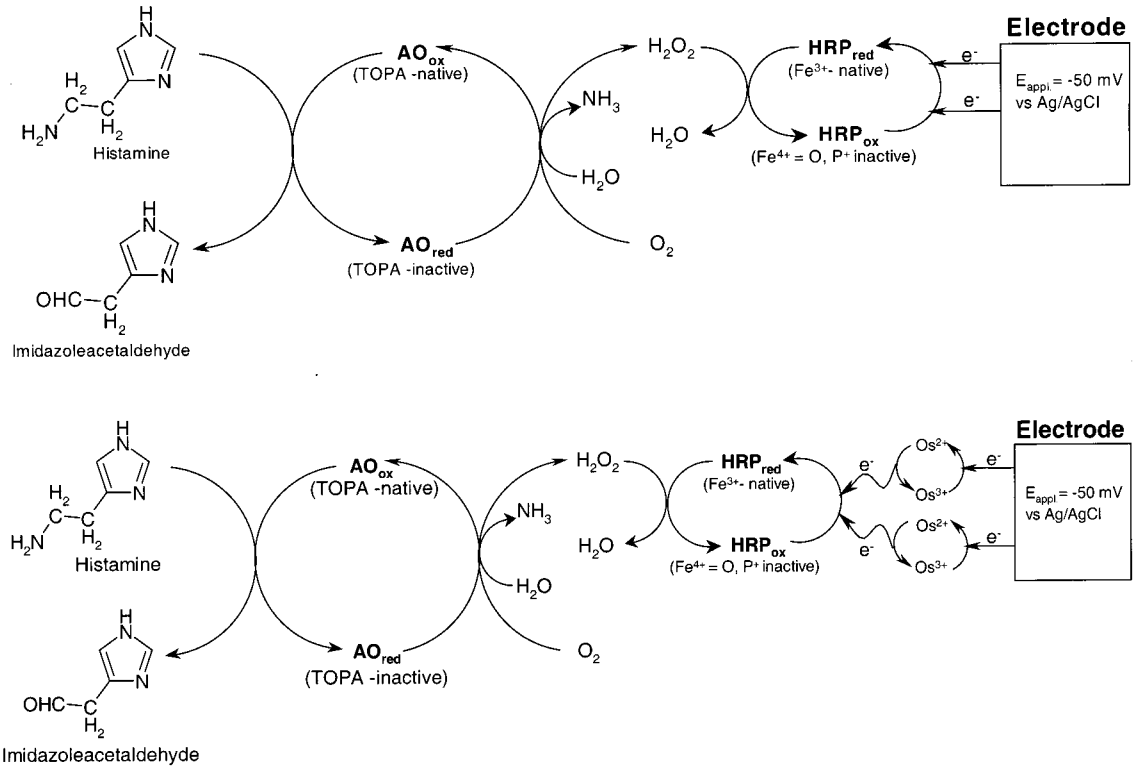

Figure 2. Scheme of the electron-transfer pathways using histamine as a model substrate for (a) direct electron transfer and (b) mediated electron transfer. Symbols: AO_{ox}, AO_{red}, HRP_{ox}, HRP_{red} are the oxidized and reduced forms of the mentioned enzymes, respectively; TOPA-inactive and TOPA-native represent trihydroxyphenylalanine and its quinonic form, being the reduced and oxidized states of the AO cofactor.

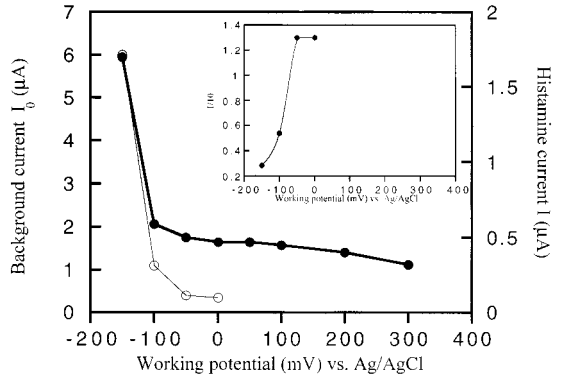

Figure 3. Hydrodynamic voltammogram recorded for type I electrodes using 100 μM histamine (●), the background current (○), and their ratio (·), respectively. Conditions: electrode structure, AO:HRP 1:1 (w/w); applied potenitial, −50 mV vs Ag/AgCl; flow rate, 0.5 mL min^{-1}.

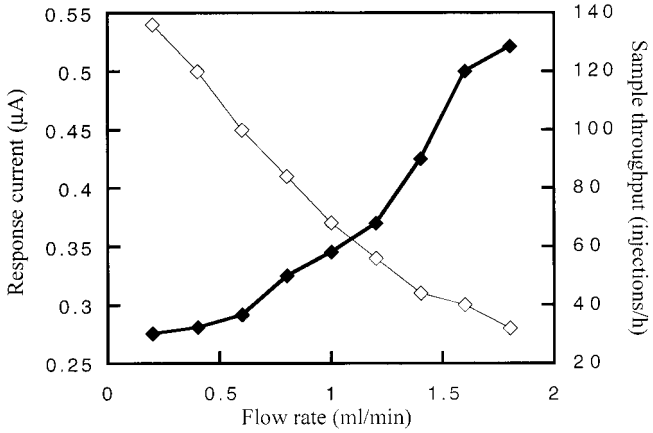

Figure 4. Effect of flow rate on current signals (□) and sample throughput (■) of type I electrodes. Conditions: 100 μM histamine; other parameters as mentioned in Figure 3.

Optimization Steps. The bienzyme electrodes were optimized with regard to several parameters, namely, working potential, flow rate, influence of various enzyme, polymer, cross-linker ratios, and electrode coating procedure.

Hydrodynamic voltammograms were recorded using 100 μM histamine as substrate and using AO−HRP-modified type I electrodes in order to establish the optimal working potential. The voltammogram, together with the ratio between the response and the background current obtained on the same condition, respectively, is shown in Figure 3. Although the response of the biosensor drastically increased when the applied potential was below −100 mV, so did the background current, demonstrating a possible oxygen electroreduction interference with the biosensing process. A potential of −50 mV vs Ag/AgCl was therefore chosen for all further experiments as a compromise between the observed response and the background currents.

The influence of carrier flow rate on the biosensor response for histamine was also considered for type I electrodes, the results being presented in Figure 4. The decrease in peak height with increasing flow rates demonstrates a limitation either due to the bioconversion of the amine substrate by AO or to the reduction of H_2O_2 by the direct electron transfer between HRP and graphite electrode. The phenomenon is not clarified yet, since the later reaction is known to be a kinetically slow process,[42] but also our recent study has indicated that the reaction catalyzed by AO is

(42) Ruzgas, T.; Csöregi, E.; Emnéus, J.; Gorton, L.; Marko-Varga, G. *Anal. Chim. Acta* **1996**, *330*, 123−138.

Table 1. Biosensor Characteristics of Type I Electrodes Obtained for Different AO:HRP Ratios (w/w%)[a]

enzyme ratio	analyte	K_m^{app} (μM)	I_{max} (μA)	S (mA/M cm^2)	C (%)	DL (μM)
AO 87%	histamine	279 ± 16	1.03 ± 0.02	50.6 ± 0.8	19.0	0.16
HRP 13%	putrescine	153 ± 15	1.96 ± 0.06	175.5 ± 1.4	66.2	0.06
	H_2O_2	93 ± 3	1.80 ± 0.21	265.1 ± 1.6		
AO 80%	histamine	332 ± 17	1.34 ± 0.03	55.3 ± 0.7	16.6	0.20
HRP 20%	putrescine	228 ± 15	3.01 ± 0.07	180.8 ± 0.9	54.7	0.07
	H_2O_2	112 ± 8	2.70 ± 0.06	330.2 ± 1.0		
AO 67%	histamine	370 ± 22	1.30 ± 0.03	48.1 ± 0.1	14.7	0.25
HRP 33%	putrescine	240 ± 15	3.10 ± 0.01	176.9 ± 0.9	54.2	0.07
	H_2O_2	153 ± 6	3.64 ± 0.04	325.9 ± 0.5		
AO 50%	histamine	437 ± 43	1.22 ± 0.04	38.2 ± 1.4	12.7	0.33
HRP 50%	putrescine	268 ± 23	3.05 ± 0.10	155.9 ± 1.3	52.0	0.08
	H_2O_2	175 ± 8	3.83 ± 0.05	299.8 ± 0.6		
AO 40%	histamine	441 ± 23	1.16 ± 0.02	36.0 ± 0.7	10.9	0.34
HRP 60%	putrescine	276 ± 22	3.69 ± 0.06	183.1 ± 1.1	55.7	0.13
	H_2O_2	206 ± 3	4.94 ± 0.03	328.5 ± 0.2		
AO 33%	histamine	479 ± 41	1.37 ± 0.10	39.2 ± 1.5	12.2	0.41
HRP 67%	putrescine	287 ± 12	3.84 ± 0.06	183.3 ± 0.6	57.0	0.08
	H_2O_2	211 ± 18	4.95 ± 0.15	321.4 ± 1.2		

[a] I_{max} and K_m^{app} values were estimated from the Michaelis−Menten equation: $I = (I_{max} \times [A])/(K_m^{app} + [A])$. S is the sensitivity (calculated as $I_{max}/K_m^{app}s$, where s is the active area of the electrode), C is the conversion efficiency (calculated as $S_{analyte}/S_{H_2O_2}$), and DL is the detection limit (calculated as $3S/N$). The linear range for all studied configurations was 1−100 μM.

Table 2. Bioelectrochemical Characteristics Obtained for Bienzyme Electrodes Based on Direct and Mediated ET[a]

electrode type	analyte	K_m^{app} (μM)	I_{max} (μA)	S (mA/M cm^2)	C (%)	DL (μM)	LR (μM)
type I	histamine	332 ± 17	1.34 ± 0.02	55.3 ± 0.7	16.74	0.16	1−100
	putrescine	227 ± 16	3.01 ± 0.07	181.6 ± 1.0	55.01	0.06	1−100
	H_2O_2	112 ± 8	2.70 ± 0.06	330.1 ± 1.0			1−100
type II	histamine	901 ± 85	4.85 ± 0.41	73.74 ± 1.7	23.07	0.33	1−150
	putrescine	512 ± 40	7.26 ± 0.53	194.1 ± 1.4	60.73	0.17	1−400
	H_2O_2	977 ± 92	22.8 ± 1.68	319.6 ± 1.6			1−250

[a] LR is linear range and the other symbols are as in Table 1.

the rate-limiting one.[38] According to the obtained results an optimal working flow rate was chosen to be 0.5 mL min^{-1}, as a compromise between the biosensor kinetics and its sample throughput.

To achieve an effective electron transfer (ET) all electrode types were optimized with regard to the ratio of the used enzymes (type I), composition of the redox hydrogel (type II), and influence of electrode coating procedures (types IIIa−d). Table 1 shows the kinetic parameters and the main biosensor characteristics obtained for type I electrodes at different ratios of AO:HRP. The increasing tendency of the apparent Michaelis constant observed for type I electrodes with the increasing amount of immobilized HRP was attributed to an increase in the thickness of the total protein loading on the electrode surface, decreasing the diffusion rate of the analytes into the film. The conversion efficiency of type I biosensors (defined as the ratio between the sensitivities for amines and H_2O_2, respectively) decreased with decreasing AO content, showing a catalytic limitation of the AO-catalyzed reaction and a first-order kinetics for the amine substrates within the dynamic range. As a compromise between response time, sensitivity, and detection limit for the two substrates, the electrodes containing 20% HRP and 80% AO were chosen as optimal for further experiments. The dynamic range for all type I biosensors was 1−100 μM for both histamine and putrescine.

To improve the electron transfer kinetics between HRP and the graphite electrode, as well as the biosensor stability, a new electrode design was considered that integrated the two enzymes into an Os-based redox polymer. Redox hydrogels have been previously shown to represent an effective matrix for enzyme immobilization, resulting in increased stability and enhanced rate of the ET.[32,43,44] It is known, however, that the rate of the ET is highly influenced by the composition of the redox hydrogel, as well as by the kinetics of the used enzymes. Therefore various biosensor preparations (types II and III) were considered in order to find the optimal electrode structure displaying the most efficient ET pathway.

The redox hydrogel based biosensors were first examined in order to determine the influence of the amount of redox polycation and the cross-linking agent in the biosensor's structure. Table 2 shows the obtained results. Increasing the number of components on the electrode surface resulted in an increased diffusional barrier, a tendency reflected in the apparent Michaelis constants (3-fold increase for histamine and 2-fold increase for putrescine). However, the introduction of the electrochemical mediator caused a considerable improvement in bioelectrocatalytic efficiency, indicated by an increased I_{max} (3-fold increase for histamine and 1,5-fold for putrescine) and sensitivity (30% and 7% increase for histamine and putrescine, respectively). The hydrogen

(43) Csöregi, E.; Schmidtke, D. W.; Heller, A. *Anal. Chem.* **1995**, *66*, 2451−2457.
(44) Larsson, N.; Ruzgas, T.; Gorton, L.; Kokaia, M.; Kissinger, P. T.; Csöregi, E. *Electrochim. Acta* **1998**, *43*, 3541−3554.

Table 3. Influence of the Electrode Coating Procedure on the Biosensor Characteristics[a]

type of electrode	analyte	K_m^{app} (μM)	I_{max} (μA)	S (mA/M cm^2)
type II	histamine	901 ± 85	4.85 ± 0.41	73.7 ± 1.7
	putrescine	512 ± 40	7.26 ± 0.53	194.1 ± 1.4
type IIIa	histamine	789 ± 35	3.56 ± 0.08	61.8 ± 0.7
	putrescine	449 ± 34	7.72 ± 0.69	235.5 ± 1.6
type IIIb	histamine	687 ± 47	2.66 ± 0.24	53.0 ± 1.5
	putrescine	473 ± 28	2.04 ± 0.13	59.1 ± 1.2
type IIIc	histamine	689 ± 33	2.17 ± 0.06	43.1 ± 0.7
	putrescine	422 ± 35	7.83 ± 0.82	254.2 ± 1.8
type IIId	histamine	649 ± 19	1.90 ± 0.02	40.1 ± 0.4
	putrescine	425 ± 24	2.14 ± 0.20	69.0 ± 1.5

[a] Symbols are as in Table I.

peroxide sensitivity remained practically unchanged, confirming previously reported results for other bienzyme hydrogel electrodes.[32] Type II electrodes displayed improved detection limit and linear range for the studied analytes. Therefore, this design (type II electrodes) was chosen for further experiments also due to improved electrode stability (results not shown).

The effect of the coating procedure was also studied. Besides coating with a premixed solution of all four components (type II), different possibilities of sequential coating of the electrode surface (type III) were considered (see Table 3). It was previously demonstrated that both HRP and AO can be electrically wired to the redox polymer and, thus, might cause a partial short circuit[32,38] when all components are mixed together. This assumption was confirmed for the main substrate (putrescine) for which an increase in sensitivity of about 30% was observed for the two-layer electrodes (type III), compared to the single-layer ones (type II). Comparing type III electrodes, it seems confirmed that IIIb type electrodes represent the worse structure for the diffusion of the substrates, resulting in the highest K_m^{app}, a value similar to that obtained for type II electrodes. Signals recorded for type IIIb and IIId electrodes were about 30% less than those recorded for types IIIa and IIIc, confirming partly that the ET is hindered when the HRP layer (wired or not) is not in direct contact with the electrode surface. Some intermixing of the layers was observed, as previously demonstrated for other enzymes such as glucose oxidase and lactate oxidase.[45] No considerable change was observed for the other substrate, histamine, the slight decrease in sensitivity being unrepresentative considering the differences of about 10−15% in similar electrode preparation. However, a certain interaction between histamine and horseradish peroxidase could be observed, causing a decreased sensitivity (see electrode type IIId) compared to type II electrodes. More studies have to be done to fully elucidate the mechanism of the ET observed for the two substrates. Clearly, the less sensitive electrode configuration is represented by type IIId electrodes, for which the bias currents due to the wiring of AO are the most explicit. Considering the simplicity of electrode preparation and the small differences in the electrode characteristics between type II and type III electrodes, type II was chosen as the optimal electrode design.

(45) Csöregi, E.; Quinn, C. P.; Schmidtke, D. W.; Lindquist, S. E.; Pishko, M. V.; Ye, L.; Katakis, I.; Hubbell, J. A.; Heller, A. *Anal. Chem.* **1994**, *66*, 3131−3138.

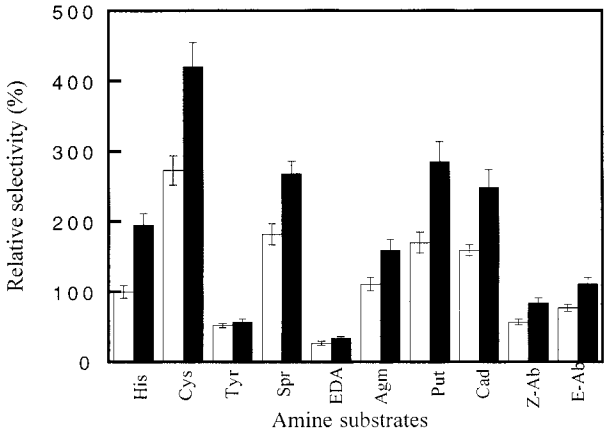

Figure 5. Relative selectivity recorded for type I (white) and type II (black) electrodes for different substrates using histamine signals obtained for type I electrodes as reference. Symbols: His, histamine; Cys, cystamine; Tyr, tyramine; Spr, spermidine; EDA, ethylenediamine; Agm, agmatine; Put, putrescine; Cad, cadaverine; *Z*,*E*-Ab, *Z*,*E*−1,4-diamino-2-butene.

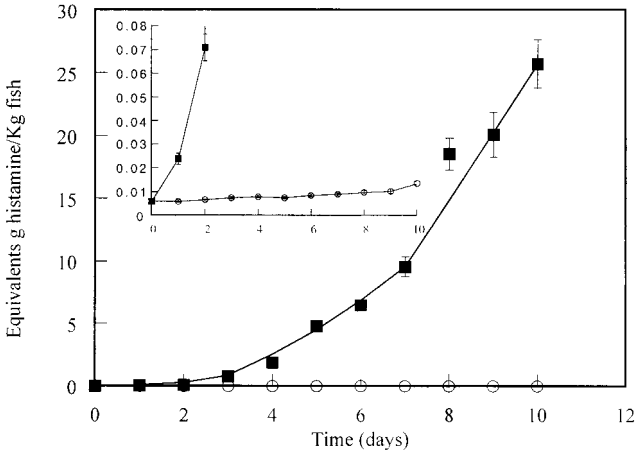

Figure 6. Monitoring of fish freshness using type II electrodes. Total amine content recorded for fish kept at 4 °C (○) and at 25 °C (■), respectively. The total amine concentration was expressed in histamine equivalent units.

Characteristics and Applications. Type II biosensors were further characterized with regard to selectivity, response time, and operational and storage stability. Figure 5 shows the relative selectivity for different AO substrates, using histamine as the reference compound, since it is considered to be a biomarker of major interest. As seen, the response for aliphatic amines is generally higher than those observed for the aromatic ones. Also, type II biosensors yielded higher sensitivities than type I ones, probably caused by better electron-transfer kinetics.

The response time of the sensor, calculated as the time elapsed between 5% and 95% of response height, was fast (less than 1 min).

The operational stability of the biosensor was studied both for histamine and putrescine as substrates. The response current of the bienzymatic enzyme electrodes decreased with about 30% and 50% for histamine and putrescine, respectively, after 10 h of continuous operation with a sample throughput of 30 injections h^{-1}. The storage stability of the electrodes was good, a decrease of only about 10% and 15% being observed for histamine and putrescine, respectively, after 10 days of storage.

The optimized biosensor was considered for monitoring biogenic amines in real samples. The differentiation between the signals given by different amines is, however, not possible, only the total amine content of the sample was determinable. Fish-muscle samples, kept for 10 days at 4 and 25 °C, respectively, were analyzed after extraction in PB by direct injection in the flow system. The total amine content expressed in histamine equivalents is presented in Figure 6. The maximum accepted limit for total amine concentration in food products is 100−200 mg kg^{-1} sample, and a concentration of 1000 mg kg^{-1} is considered to be toxic.[6] After 3 days of storage at room temperature, the fish became improper for consumption, while even after 10 days of storage at 4 °C no major changes could be observed in the total amine concentration.

CONCLUSIONS

The present work shows the development, optimizations, and possible application of bienzyme electrodes for the determination of biogenic amines. The developed electrodes are based on an amine oxidase, which is readily producible with high yield, and were based either on a direct or on a mediated electron-transfer approach. The optimized redox hydrogel incorporated bienzyme electrodes were characterized by high sensitivity, good operational and storage stability, fast response time, and low detection limit, making them very promising for food quality assessing. As previously demonstrated, a biosensor with only AO immobilized on a graphite electrode is selective for histamine, cystamine, and tyramine but not for cadaverine and putrescine, while the bienzyme sensors described in this work are selective for all of them. Thus, using a combination of the two developed biosensors the separation of the signals given by the mentioned biogenic key amines is possible.

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