DISSERTATIONES CHIMICAE UNIVERSITATIS TARTUENSIS
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Diamine oxidase-based biosensors: construction and working principles

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“Science is a great game. It is inspiring and refreshing.
The playing field is the universe itself.”

Isidor Isaac Rabi (1898–1988)

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LIST OF ORIGINAL PUBLICATIONS

The present thesis consists of five original research papers and a review, which are referred in the text by Roman numerals I–VI.

I. **Kivirand, K.,** Rinken, T., Purification and properties of amine oxidase from pea seedlings. Proceedings of the Estonian Academy of Sciences Chemistry, 56 (2007) 164–171.

II. **Kivirand, K.,** Rinken, T., Interference of the Simultaneous Presence of Different Biogenic Amines on the Response of an Amine Oxidase Based Biosensor. Analytical Letters, 42 (2009) 1725–1733.

III. Rinken, T., Rinken, P., **Kivirand, K.** Signal Analysis and Calibration of Biosensors for Biogenic Amines in the Mixtures of Several Substrates. In: Biosensors for Health, Environment and Biosecurity, Book 1. ISBN 978-953-307-155-8. InTech – Open Access Publisher (2011) (In Press).

IV. **Kivirand, K.,** Rinken, T., Preparation and Characterization of Cadaverine Sensitive Nylon Threads. Sensor Letters, 7 (2009) 580–585.

V. **Kivirand, K.,** Rebane, R., Rinken, T., A Simple Biosensor for Biogenic Diamines, Comprising Amine Oxidase – Containing Threads and Oxygen Sensor. Sensor Letters (2011) (Article in Press).

VI. **Kivirand, K.,** Rinken, T., Biosensors for Biogenic Amines: the Present State of Art Mini-review. Analytical Letters, (2011) (Article in Press).

Author's contribution

Paper I: Performed all the experimental work and calculations, responsible for the interpretation of results and writing of the paper.

Paper II: Performed all the experimental work and calculations, responsible for the interpretation of results and writing of the paper.

Paper III: Performed all the experimental work.

Paper IV: Performed all the experimental work and all calculations, responsible for the interpretation of results and writing of the paper.

Paper V: Performed all the experimental work and calculations, responsible for the interpretation of results and writing of the paper.

Paper VI: Responsible for writing of the paper.

ABBREVIATIONS AND SYMBOLS

A	total possible biosensor signal change
A_{max}	theoretical maximum of signal change
AGM	agmatine
AO	amine oxidase (EC 1.4.3.6 till 2008)
APTES	3-aminopropyltriethoxy-silane
AUH	agmatinase (EC 3.5.3.11)
B	initial maximal slope of the enzyme-catalyzed process curve
BA	biogenic amine
BAT-silasorb	(2-[4,6-bis (aminoethylamine)-1,3,5-triazine])-Silasorb
BSA	bovine serum albumin
c_s^{bulk}	substrate concentration in solution
CAD	cadaverine
CE	capillary electrophoresis
CNT	carbon nanotube
CPG	aminopropyl controlled pore glass
CYS	cystamine
DAO	diamine oxidase (EC 1.4.3.22 from 2008)
DEAE	diethylaminoethyl
DET	direct electron transfer
DMS	dimethyl sulfate
$[E]_{total}$	overall concentration of the enzyme
EDAC	1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (hydrochloride)
EEC	European Economic Community
FDA	Food and Drug Administration
FIA	flow injection analysis
FMO3	flavine-containing monooxygenase type 3 (EC 1.14.13.8)
GA	glutaraldehyde
GC	glassy carbon
GMBS	γ-maleimidobutyric N-hydroxysuccinimide ester
HEMA	2-hydroxyethyl metacrylate
HIS	histamine
HPLC	high performance liquid chromatography
HRP	horseradish peroxidase (EC 1.11.1.7)
$I_{(t)}$	biosensor output current at time moment
I_0	output current at the start of the reaction
IMS	ion mobility spectrometry
k_{cat}^*	apparent catalytic constant of the enzyme-catalyzed reaction
$k_{dff}^{O_2}$	apparent diffusionconstant of oxygen

K_{O_2}	dissociation constant for the enzyme-oxygen complex
K_S	dissociation constant for the enzyme-substrate complex
$K_{1/2}$	half-signal change constant
K_M	Michaelis-Menten constant
MADH	methylamine dehydrogenase (EC 1.4.99.3)
MAO	monoamine oxidase (EC 1.4.3.21 from 2008)
MRL	maximum residue level
NHS	N-hydroxysuccinimide
PAO	polyamine oxidase (EC 1.5.3.14)
PDDA	(diallyldimethylammonium) chloride
PEGDGE	poly(ethylene glycol) (400) diglycidyl ether
PO	putrescine oxidase (EC 1.4.3.10)
PPYox-PβNAP	poly-pyrrole poly-β-naphtol
PSAO	pea seedling amine oxidase
PUT	putrescine
PVA-SbQ	polyvinyl alcohol containing stilbazolium groups
$PVI_{13\ or\ 7}$-dmeOs	poly(1-vinylimidazole) with [osmium(4,4´-dimethylbipyridine)$_2$Cl$^{+/+2}$]
R^2	squere of the correlation coefficient
SNHS	suberic acid bis(N-hydroxysuccinimide ester)
SPD	spermidine
SPE	screen-printed electrode
SPM	spermine
SPP	sweet potato peroxidase (EC 1.11.1.7)
TLC	thin-layer chromatography
TMA	trimethylamine
TRY	tryptamine
TYR	tyramine
UV	ultraviolet
τ_s	inertia of the transducer´s response
σ	standard deviation

INTRODUCTION

Recent trends in food science are promoting an increasing search for trace compounds that can affect human health. Biogenic amines are natural amines produced during decarboxylation of amino acids or by amination and transamination of aldehydes and ketons, belong to this group of substances. The amounts of biogenic amines are usually increased during controlled or spontaneous microbial fermentation of food or in the course of food spoilage. The most common biogenic amines, used for the indication of food quality, are histamine, cadaverine and putrescine. At present, regulations have been established only for the intake of histamine, although several studies have indicated undesirable effects of other biogenic amines.

A prospective option for a rapid on-line detection of biogenic amines is the application of biosensors. The benefits of biosensors are their low cost, short analysis time, simplicity and possibility to be used outside an organized laboratory. In order to obtain biosensor systems for the detection of biogenic amines, several amine-selective enzymes have been employed. As the selectivity of these enzymes is usually quite low, problems arise with the detection in the mixtures of several biogenic amines as potentially interfering compounds. Concerning the detection of histamine, this may cause serious underestimation of histamine levels in the presence of other biogenic amines. Therefore one of the aims of the present study is to characterize the catalytic activity of diamine oxidase, isolated from pea seedlings, towards different amines in their "monosolutions" and mixtures. Based on the obtained data, multivariate models are proposed for the calibration of biosensors in the presence of several biogenic amines.

Besides selectivity, operational stability is the other crutial problem for the construction of biosensing systems, as the inactivation of the biosensor biorecognition element decreases the sensitivity of the sensor and leads to the need of its frequent recalibration. In the present work the problems of stabilization of diamine oxidase are examined by optimizing different methods of its covalent immobilization onto nylon carriers.

The main purpose of present thesis is to develop a simple, flexible and selective pea seedling amine oxidase based biosensor for a rapid detection of biogenic amines.

1. LITERATURE OVERVIEW

1.1. Short characterization of amine oxidases

Amine oxidases (AOs, previously copper-containing amine oxidase EC 1.4.3.6, in 2008 EC entry deleted and replaced by monoamine oxidase (MAO) EC 1.4.3.21 and diamine oxidase (DAO) EC 1.4.3.22) catalyze the oxidative deamination of primary amines [1–6]. The reaction catalyzed by AOs follows a classic ping-pong mechanism composed of distinct half-reactions. The catalytic mechanism can be divided into two half-reactions, namely enzyme reduction by the substrate (Eq. 1) followed by enzyme re-oxidation by molecular oxygen (Eq. 2) [2,6–9]:

$$E_{ox} + R - CH_2 - NH_3^+ \rightarrow E_{red} - NH_3^+ + R - CHO \quad (1)$$

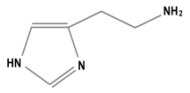
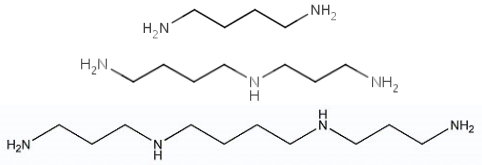
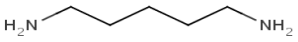
$$E_{red} - NH_3^+ + O_2 + H_2O \rightarrow E_{ox} + NH_4^+ + H_2O_2 \quad (2)$$

AOs are isolated from tissues of various mammals and plants: plant enzymes with amine oxidase activity have been purified mainly from Leguminosae (mostly from lentil and pea seedlings) [10–12]. Data of AOs' substrate specificity varies in a wide range depending on enzyme origin and seems to be dependent even on the experimental method applied [13,14].

Pea seedling amine oxidase (PSAO) has been found to be selective towards short aliphatic diamines like putrescine (1,4-diaminobutane) and cadaverine (1,5-diaminopentane). The relative specific activity of PSAO has been found to be 100% towards putrescine, 111% towards cadaverine, 56% towards agmatine and spermidine, 44% towards 1,6-diaminohexane, 30% towards histamine, 8% towards spermine [14]; no activity has been detected towards 1,3-diaminopropane [15].

1.2. Biogenic amines

Biogenic amines (BAs) are basic nitrogenous compounds formed in the process of microbial decarboxylation and aging of free amino acids [16–19]. Precursors and chemical structures of the most common biogenic amines are given in Scheme 1. The BA content has been applied as a parameter for the evaluation of food quality. Because of significant variations in the amino acid composition of different products, the nature and amount of BAs formed can vary in a wide range; therefore, levels of different BAs or their combinations have been proposed as indicators of spoilage in different foodstuff [20].

Precursor Amino Acid		Biogenic Amine	
Histidine	→	Histamine	
Arginine ↓ Ornithine	→	Putrescine ↓ Spermidine ↓ Spermine	
Lysine	→	Cadaverine	

Scheme 1. Precursors and chemical structures of the most common biogenic amines.

The most hazardous compound among BAs is considered to be histamine [18,21,22]. The level of histamine toxicity depend on the efficiency of the detoxification system of a body [22]. In the case of the histamine alone, the detoxification system eliminates histamine by specific intestinal histamine-metabolizing enzymes such as diamine oxidase. However, in the presence of other BAs (such as cadaverine and/or putrescine) histamine-metabolizing enzymes are inhibited [16,19,23,24].

The combined level of putrescine and cadaverine has been suggested to serve as an index of food acceptability, because the concentrations of these amines increases considerably prior to spoilage and correlate well with the microbial load [25–27].

At present, regulations have been established only for the intake of histamine, but no limits have been set for other BAs, including putrescine and cadaverine. The allowed maximum residue level (MRL) of histamine in food according to European Economic Community (EEC) regulations is 100 mg/kg [28]; the U.S. food safety organization Food and Drug Administration (FDA) has established a level two times lower: 50 mg/kg [29].

1.3. Detection of biogenic amines

The determination of amines in food is important for their effect on human health and the potential action as markers of food quality [16,30–34]. Several methods have been reported for the analysis of BAs [18,35]. Traditionally BAs are determined with different chromatographic methods. The most common analytical tool has been high performance liquid chromatography (HPLC),

allowing low detection limits (below 0.06 ppb) [36–40], but being time-consuming and requiring special instrumentation. Thin-layer chromatography (TLC) is simple and easy to carry out, but the obtained results allow only semi-quantitative analyses [41,42]. Capillary electrophoresis (CE) combined with fluorescence detection (as the sensitivity of electrochemical and ultraviolet detectors is lower) has also been widely used for the detection of BAs [43,44].

The largest drawbacks in the analysis of BAs in food are the complexity of the sample matrix and the low concentration levels at which the compounds are present in the samples. Therefore, extraction and purification steps, enabling to remove interfering compounds from the matrix, are unavoidable prior to chromatographic analysis. Following these steps, BAs are commonly converted to derivatives, since the majority of biogenic amines do not possess chromophobic or fluorogenic moieties theirselves. In addition, aliphatic amines have low optical absorbance in the ultraviolet (UV) region and therefore a derivatization procedure is required also prior to UV detection. Alternative options for BA detection are conductiometry [44,45], amperometry [46,47] and ion mobility spectrometry (IMS) [48]. All these methods, requiring extensive sample clean-up, lead to low sample throughput and are therefore not suitable for *on-line* monitoring of food quality.

I.3.1. Biosensors for biogenic amines

The application of biosensors for BA analyses is a good alternative to traditional methods, as for low cost, short analysis time, simplicity and possibility to be used outside an organized laboratory. In order to obtain selective biosensor systems for BAs, different enzyme-catalyzed reactions have been employed; alternative biorecognition systems are not known [VI].

In biosensors the enzyme specificity is combined with the analytical precision of signal transduction of the biorecognition reaction. The choice of the transducing system depends mostly on the enzyme(s) employed and the success of an enzyme biosensor lies on the quality of the enzyme-sensor linkage [49]. Five different enzymes: monoamine and diamine oxidase (previously amine oxidases), putrescine oxidase (PO, EC 1.4.3.10), methylamine dehydrogenase (MADH, EC 1.4.99.3) and flavin-containing mono-oxygenase type-3 (FMO3, EC 1.14.13.8) have been used for the generation of BA specific biorecognition in biosensors.

The biosensor analyses are usually based on the steady state response of measuring system, where the system generates the maximum response and it is commonly claimed that the sensitivity of biosensor systems towards particular amines is not altered by other amines present in the sample [50]. However in studies conducted by Albert-Ruiz (1999) the measured additive absorbances of putrescine, cadaverine and histamine were about 10% smaller than their expected values. According to the presented data, the absorbances were smaller in all cases where putrescine and/or cadaverine were present [51].

The most common signal transducers for the construction of BAs biosensors are various electrochemical sensors, which can be classified into three generations according to the electron transfer mechanism utilized [52,53]. For the quantification of BAs measurements of oxygen consumption or hydrogen peroxide production are carried out in the biosensors of 1st generation [54,55]. To minimize the effect of oxygen concentration in probe and to cut the working potential, artificial redox mediators have been used for the construction of BA biosensors of the 2nd generation [52,56–61]. Biosensors, based on direct electron transfer (DET) between the active site of an enzyme and electrode surface and enabling measurements in potential ranges close to the redox potentials of enzymes theirselves, are known as the 3rd generation biosensors [24,52,53,62–65]. An overview of different biosensors, used for the detection of BAs in food, is given in Table 1 [VI]. These biosensors are all based on combinations of some of the abovementioned amine-selective enzyme and an electrochemical signal transduction system. The detection limits of different biosensors for BAs are varying from 0.05 ppb (0.0005 μM) to 10 ppm (100 μM) and their lifetime from disposable biosensors [56] to biosensors, enabling to carry out measurements for at least 8 months [66] (Table 1).

Table 1. Currently studied biosensors for the detection of different biogenic amines.

Biogenic amines	Biosensor construction and biocomponent	Application in food	Linear range (μM)	Detection limit (μM)	Stability	Ref.
CAD, PUT	Nylon/DMS-GA-DAO	Fish	up to 300	60–100	3 months at 4°C; 1 month for daily operations	[67]
HIS, PUT, CAD	Pt/cellulose acetate/nylon net-DAO-GA/polycarbonate	Fish	1–50	0.5	–	[68]
PUT	HiTrap™ NHS-activated minicolumns/AO-HRP (batch)	Fish	0.0009–0.07	0.0005	Average lifetime 20 days	[69]
HIS, PUT	Silanized Pt/GA-AO	Fish	0.17–20	–	–	[70]
PUT	Pt/AH Sepharose-BSA-GA-AO (batch)	–	0.5–10	0.2	Stable for more than 32 hours of continuous operation at room temperature	[71]
PUT, CAD	Pt/PPYox-PβNAP-DAO-GA (FIA)	Cheese and anchovy	500	6–12	13% of sensitivity loss after 3 weeks of continuous operation	[72]
PUT, CAD	Pt/Immunodyne™ membrane-GA-AO (batch)	–	up to 6000	25	20% and 50% of sensitivity lost in 2 mo at 5°C and room temperature, respectively	[73]
PUT, HIS, TYR	Carbon paste (HRP, ferrocene, BAT-silasorb)/DAO (batch)	–	up to 100	25	1 week at 4°C, 50% of sensitivity lost in 14 days at 37°C	[74]
PUT, CAD	Pt/cellulose acetate-DAO-GA (batch)	Fish	1–100	0.6	1 week (80% of sensitivity lost in 7 days)	[75]
PUT, CAD, HIS	Aminopropyl glass/GA-AO	–	up to 60	0.5	70% of initial activity after 1 month	[50]
CAD, PUT	Graphite/DAO, HRP, PVI13-dmeOs, PEGDGE (FIA)	Fish	up to 5	–	After 4 h of continuous operation at room temperature, the decrease of the overall biosensor sensitivity was 0.41% (for Cad)	[57]
PUT	Pt disks/GA-Nafion membrane-PO	Meat	5–60	–	–	[76]

Table 1. Continued

Biogenic amines	Biosensor construction and biocomponent	Application in food	Linear range (μM)	Detection limit (μM)	Stability	Ref.
PUT	Pt/Immonodyne^{TM} membrane-GA-BSA-DAO (batch)	Fish	up to 6000	25	2 months at 5°C (60 assays)	[77]
PUT, CAD	Polypropylene tube/chitosan porous beads-GA-PO	Chicken	up to 1000	10	8 months at 4°C; usable for at least 300 assays	[66]
PUT, CAD	Micro gold thin-film electrode/GA-BSA-PO (FIA)	Fish	100–1000	10	Continuous operation over a period of 2 weeks	[78]
PUT[(a,b)], SPM[(b)], SPD[(b)]	[(a)]Pt/cellulose acetate membrane/nylon-GA-DAO/polycarbonate membrane [(b)]Pt/cellulose acetate membrane/Immobilon membrane-PAO/polycarbonate membrane	Fruits	[(a)] 2–2000 [(b)] 2–1000	1	The sensitivity of DAO sensor remained unchanged for 10 days of continuous analysis; the lifetime of PAO sensor was almost 45 days	[79]
HIS[(a,b)]	[(a)]Graphite/DAO [(b)] Graphite/DAO, HRP, Os-hydrogel, PEGDGE (FIA)	Fish	[(a)] 10–100 [(b)] 10–200	[(a)] 2.7 [(b)] 2.2	Stability loss of 20% during 8 h continuous operation (30 injections per h)	[24]
PUT[(a)], HIS[(b)], TYR[(c)]	Pt/GA-BSA-AO	Fish; cheese; meat; vegetables; wine; beer	[(b,c)] up to 1800 [(a)] up to 1000	[(b,c)] 70 [(a)] 50	The sensors were stable for only 1 week (remaining activity: 80% after 7 days)	[80]

Table 1. Continued

Biogenic amines	Biosensor construction and biocomponent	Application in food	Linear range (μM)	Detection limit (μM)	Stability	Ref.
PUT	Glass beads/GA or SNHS or GMBS or EDAC-HRP-PO (FIA)	Fish	up to75	5	After 600 injections the response remained 75% of its initial value	[81]
HIS, PUT, CAD, TYR, SPD, SPM	Carbon/DAO-HRP (SPE)	Fish	up to 1.6	0.18	Disposable biosensor	[56]
HIS, PUT, CAD	Carbon paste/photoHEMA-DAO (SPE)	Fish	up to 600	7	The lifetime was up to one month at 4°C	[82]
HIS, TYR, PUT, CAD, TRY	Pt/cellulose acetate/nylon net-DAO-glutaraldehyde/polycarbonate (FIA)	Cheese	up to 1000	1	A decrease of 60% of initial activity after 1 month (at 4°C)	[83]
AGM, PUT, CAD, SPM	CPG/GA-AUH-PO	Fish	up to 1000	5	Stable for up to 50 measurements a day for 4 days	[84]
PUT	Graphite electrode/HRP or SPP-AO-PVI_7-dme-Os-/PEGDGE (FIA)	–	up to 500	0.3–0.5	The biosensors retained 30% (SPP) and 35% (HRP) of their initial response after 400 min	[58]
HIS	Gold/MADH-PPY-ferricyanide (batch)	–	up to 500	25	–	[59]

Table 1. Continued

Biogenic amines	Biosensor construction and biocomponent	Application in food	Linear range (μM)	Detection limit (μM)	Stability	Ref.
HIS	GC/(αF55A)MADH-PPY-ferricyanide (batch)	–	–	5	Electrodes were stored dry for 2 weeks at 4°C. The mean value of the percent of current response of the electrode after storage was within 1% of that of fresh electrodes	[60]
HIS[(a)], CYS[(a)], TYR[(a)], CAD[(a,b)], PUT[(a,b)]	[(a)]Graphite electrode/AO [(b)]Graphite/HRP, Os-hydrogel-PEGDGE-AO (FIA)	Fish	[(a)] 1–100 [(b)] 1–400	[(a)] 0.06 [(b)] 0.17	[(a)]– [(b)]50% decrease after 10 h continuous operation (30 injections/h)	[34]
PUT	GC/CNT, APTES, Nafion-GA-PO	–	up to 250	0.5	–	[63]
PUT, CAD, HIS, SPM, SPD	GC/CNT, PDDA, APTES, PO/Nafion	–	up to 250	5	–	[64]
TMA	Gold/GA-BSA-FMO3	Fish	up to 670	33	The long-term storage was approximately 8 days. But the decline of the biosensor response was about 60% after 10 days.	[85]
TMA	Silicon/nylon/technicon Chemicals Co membrane/FMO3- PVA-SbQ	Fish	–	–	–	[55]

2. EXPERIMENTAL

2.1. Purification of diamine oxidase

Diamine oxidase was isolated from seedlings of *Pisum sativum* as described in [I] and used in the form of extract in 0.1 M phosphate buffer (pH 7.0) (PSAO activity 7.55 mg protein/ml and 5.43 IU of AO/mg solid). The seedlings were collected from seeds, germinated 8 days in dark at room temperature. The seedlings were homogenized and centrifuged and proteins precipitated with ammonium sulfate; the obtained enzyme-containing extract was purified with ion-exchange chromatography and gel filtration. All enzyme purification steps were carried out at 4°C and the enzyme activity was estimated after every step. The pure enzyme in solution was stable for long periods of time (no detectable loss of PSAO activity within one year) at –18°C.

2.2. Kinetic measurements and data processing

All kinetic measurements were carried out under continuous stirring in airtight glass cells in air-saturated solutions at 25°C. In experiments with soluble enzyme, the reaction was started by injecting the PSAO solution into reaction mixture; in measurements with immobilized enzyme, the PSAO-containing thread was coiled onto the oxygen sensor [V] and the reaction was started by injecting the solution of substrate(s). A cylindrical Clark-type oxygen sensor (covered with 5.65 cm^2 of 25 μm thick polyethylene film), which output current depends linearly on the dissolved oxygen concentration, was used as a basic device for monitoring the change of oxygen content in solutions due to the enzyme-catalyzed reaction(s).

The sensor output was registered automatically at 1 sec intervals until the average signal change was less than 2% in 200 seconds. The reaction characteristic parameters were calculated using the dynamic model of oximeter-based biosensors (see chapter 2.2.1) [86] and used for the characterization of enzyme properties; but also for multivariate modelling of diamine oxidase-based biosensors output in BA mixtures.

2.2.1. Basic principles of the dynamic biosensor model

The dynamic model for amperometric biosensors takes into account the ping-pong mechanism kinetics of enzyme-catalyzed reactions, diffusion of substrates and inertia of diffusion-limited sensors. It enables the calculation of steady state parameters from the biosensor transient response with errors less than 3% [86]. According to this model the normalized biosensor output current $I(t) = I_0$ is expressed as a three-parameter function of time t:

$$\frac{I(t)}{I_0} = A\exp(-Bt) + (1-A) - 2A\sum_{n=1}^{\infty}(-1)^n \frac{\tau_s}{n^2/B - \tau_s} \times \left[\exp(-Bt) - \exp(-n^2 \frac{t}{\tau_s})\right] \quad (3)$$

In Eq. 3, *I(t)* is the biosensor output current at time moment *t*; I_0 is the output current at the start of the reaction. Parameters *A* and *B* are complex parameters, both depending hyperbolically on substrate concentration: parameter *A* corresponds to the total possible biosensor signal change in case time $t \to \infty$ (normalized signal change at steady state, the maximum value of *A* equals to 1) and parameter *B* is the kinetic parameter (the initial maximal slope of the enzyme-catalyzed process curve); τ_s is the time constant of the internal processes of oxygen transducer taken together, characterizing the inertia of the transducer's response [86]. Parameters *A*, *B* and τ_s are independent of each other.

2.2.2. Multivariate signal analysis

The biosensor data (the values of maximum signal change parameters) was obtained over a longer period from experiments, carried out with different PSAO-based biosensors in the mixtures of cadaverine, putrescine and/or histamine, which concentrations varied from 0 to 3.0 mM. For correlation analysis with different models the results of overall 112 measurements were used.

2.3. Enzyme immobilization and biosensor construction

For the optimization of PSAO immobilization, the enzyme was covalently attached to nylon-6,6 threads, applying three different methods: (i) with dimethyl sulfate (DMS); (ii) with glutaraldehyde (GA); (iii) nylon carrier processed with DMS and GA successively (Scheme 3) [IV]. For the construction of a biosensor, fragments of the PSAO-containing thread with length varying from 30 to 130 cm were cut and coiled spirally around the outer surface of the cylindrical Clark-type oxygen sensor [V].

2.4. Application and validation of pea seedling diamine oxidase-based biosensors

A biosensor, comprising of 90 cm of PSAO-containing thread, was used for BAs analysis in fish (hake) probes. Hake probes (purchased from a local supermarket) were smashed, homogenized and diluted with 0.1 M phosphate buffer (pH 7.0) and used directly without any further treatment [V].

For HPLC analyses, used for the validation of biosensor results, hake probes were prepared, derivatized and analysed as described in [V]. The HPLC assessments were based on double determination of the biogenic compounds in eight samples with one week interval.

3. RESULTS AND DISCUSSION

3.1. Purification and catalytic properties of diamine oxidase

The enzyme diamine oxidase was isolated from pea seedlings (pea seedlings' amine oxidase, PSAO) applying different steps of purification. For the optimization of the germination period of the starting material, the activity of PSAO extract towards 1,5-diaminopentane (cadaverine, CAD) for different harvesting periods was studied (Fig. 1). The maximum activity was always achieved on day eight, although the absolute values of the specific activity were different in different experimental lots. Thus, the seedlings harvested on day eight of the germination were always used for further enzyme purification and kinetic measurements.

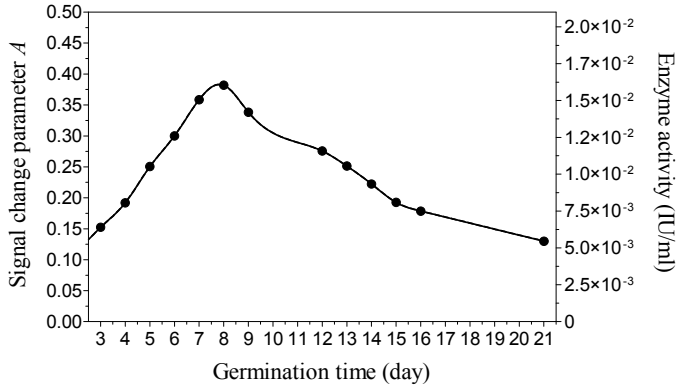


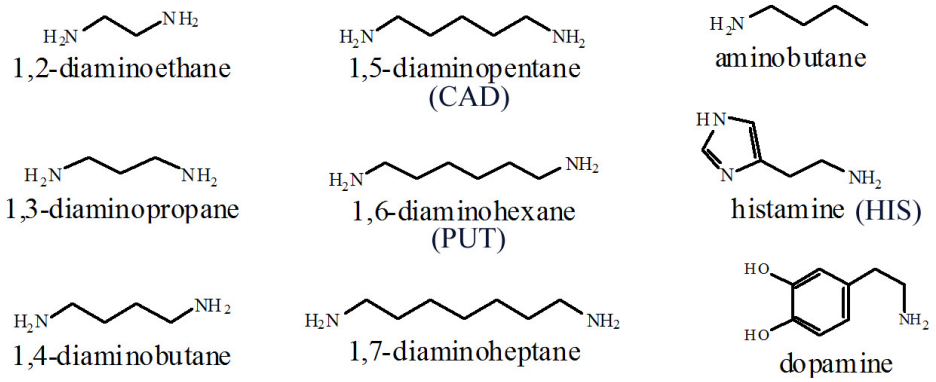
Figure 1. The specific activity of PSAO towards 0.15 mM 1,5-diaminopentane in pea seedlings extract during the germination period. Kinetic measurements were carried out in air-saturated 0.1 M phosphate buffer (pH 7.0) at 25°C.

The crude homogenate was centrifuged and fractionated with $(NH_4)_2SO_4$, which at 30% saturation enabled removal of contaminating proteins, whereas the enzyme was precipitated at 70% $(NH_4)_2SO_4$ saturation. The enzyme was further purified with ion exchange chromatography on DEAE-cellulose and collected as a passing fraction that was directly loaded on a Sephadex G-200 column. The molecular mass estimated by gel filtration was 184.0 ± 2.6 kDa. Purification efficiency at different stages was characterized as PSAO specific activity per 1 mg enzyme preparation and the percentage of recovery of the total PSAO activity. The total protein amount was determined spectrophotometrically using the method of Lowry. The purification efficiency of PSAO is shown in Table 2.

Table 2. Characterization of the purification steps of diamine oxidase from pea seedlings.

Purification step	Total activity (IU)	Specific activity (IU/mg)	Recovery (%)
Centrifuged homogenate	84.55	0.51	100
Fractionation with $(NH_4)_2SO_4$ (30%)	79.40	0.52	93.9
Fractionation with $(NH_4)_2SO_4$ (70%)	68.66	0.75	81.2
Ion exchange chromatography	48.44	0.80	57.3
Gel filtration	43.83	0.83	51.8

The activity of PSAO was studied towards various amines (Scheme 2). There was no significant difference in the qualitative catalytic properties of different PSAO preparations.



Scheme 2. Chemical structures of the amines studied.

PSAO showed the highest activity towards CAD. The maximal sensor signal change, corresponding to parameter *A* (chapter 2.2.1) for CAD was 0.76 or 76% of the total working range of the sensor. Taking the PSAO specific activity towards CAD as a standard, the PSAO selectivity towards other amines as a percentage of this value was calculated. Michaelis-Menten constant (K_M) values for each substrate were calculated relating the signal change parameter *A* with substrate concentration (Table 3).

Table 3. PSAO specific activity and K_M values for the studied amines.

Substrate	Specific activity (%)*	K_M (mM)
1,2-diaminoethane	6.4	0.022 ± 0.007
1,3-diaminopropane	8.2	0.368 ± 0.143
1,4-diaminobutane	86.2	1.150 ± 0.161
1,5-diaminopentane	100.0	1.919 ± 0.421
1,6-diaminohexane	42.6	0.321 ± 0.067
1,7-diaminoheptane	37.7	0.204 ± 0.026
histamine	9.8	0.052 ± 0.015
dopamine	11.6	0.032 ± 0.001
1-aminobutane	9.8	0.159 ± 0.064

*The activity of PSAO is expressed as a percentage of the maximum signal change towards the most efficient substrate.

The PSAO specific activity was clearly dependent on the number of methylene groups in diamine compounds and the binding of smaller and longer than 1,4-diaminobutane and 1,5-diaminopentane compounds was hindered, which may be caused by the steric hindrance in the enzyme active centre. The PSAO activity towards 1-aminobutane, which has the same number of methylene groups as 1,4-diaminobutane, was almost ten times smaller than for 1,5-diaminopentane, indicating also the importance of chemical interaction during substrate binding. The activity towards studied aromatic amines – histamine (HIS) and dopamine was in the range of 10% of activity with 1,5-diaminopentane. The high selectivity of PSAO towards 1,4-diaminobutane and 1,5-diaminopentane enables the application of the enzyme as a bioselective element for the detection of these diamines [I].

The obtained values of K_M for PUT and CAD were 1.15 ± 0.16 and 1.92 ± 0.42 mM, respectively. These values indicate that there can be additional diffusion barriers for oxygen molecules to reach the enzyme-substrate complex, which may become a limiting stage in fast processes measured with sensors.

3.2. Biosensing in the mixtures of several amines

An important requirement for biosensors with respect to their application in food samples is their indifference towards interfering compounds. As described earlier the selectivity of PSAO used in the present study, was characterized with the normalized signal change parameter *A*, calculated from the signal of an amperometric oxygen sensor, following the decrease of oxygen concentration resulting from the oxidation of a particular substrate. The dependences of the value of parameter *A* on the concentrations of CAD, PUT and HIS in single-substrate solutions are shown in Fig. 2, curves a-c.

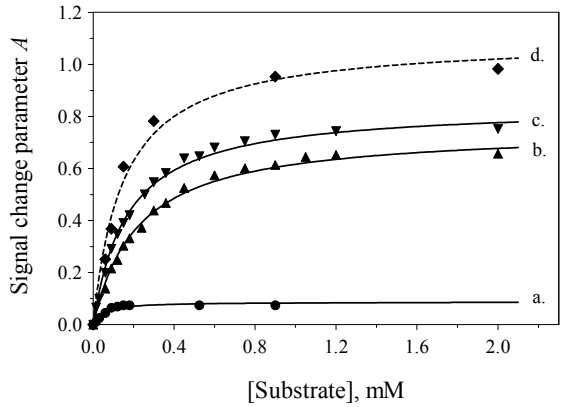


Figure 2. The calculated signal change parameter *A*: (a) HIS; (b) PUT; (c) CAD; (d) CAD and PUT in equimolar mixture. Measurements were carried out in 0.1 M phosphate buffer (pH 7.0) at 25°C, [PSAO] = 0.108 IU/ml.

The maximum values of these hyperbolic curves at high substrate concentrations indicate the activity of PSAO to particular amines. The selectivity of PSAO determines the relative speed of oxidation of different amines and has to be taken into consideration if PSAO is exploited for biorecognition for analytical purposes in solutions that simultaneously contain several biogenic amines.

The biosensor signal parameters in mixtures containing CAD and PUT were determined to study the interference of these substrates in the biosensor response. In equimolar solutions of these two diamines, the resulting signal change parameter *A* value was considerably higher than the parameter *A* values of CAD and PUT by themselves, but lower than the sum of the signal change parameter values of single substrates at similar concentrations (Fig. 2, curve d). By comparing the parameter *A* values, obtained from solutions containing only CAD or PUT and from solutions of their different mixtures, it was found that, as an average, the values of parameter *A* for mixtures were 1.14 ± 0.02 times smaller than the summed parameter *A* values for single substrates (Fig. 3). The analysis of the values of parameter *A* in mixtures at different substrates concentration rates showed that neither CAD nor PUT had a 100% impact on the signal change parameter *A* value. Both these substrates had an impact between 60 and 85% of the maximum effect at a definite concentration and the impact was smaller at concentrations below their particular K_M value. It is also interesting to notice that the effect of a single substrate was not dependent on the concentration of the other; for example, the putrescine effect in the resulting value of signal change parameter *A* in the mixture was not dependent on the CAD concentration [II].

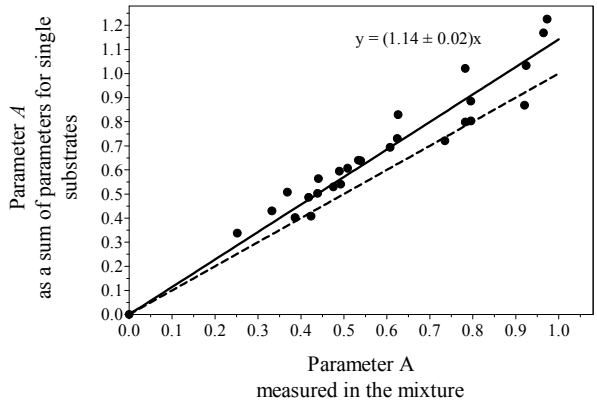


Figure 3. The correlation between the signal change parameter *A* as the sum of CAD and PUT signals and the same parameter, measured in the mixture of CAD and PUT. The dottled line marks the ideal correlation (x = y).

The biosensor response was also studied in mixtures that contained histamine (HIS) in addition to CAD and/or PUT. In cases in which the solution contained CAD and HIS, even at low CAD concentrations, it was not possible to detect any HIS effect on the response parameter *A* (Fig. 4).

The calculated values of parameter *A* were constant at fixed CAD concentrations, even if the HIS concentrations in the mixture varied from 0.01 to 3.0 mmol/l, the latter being more than 20 times greater than HIS K_M value and exceeding several times the HIS MRL in foods allowed by EEC regulations (100 mg/kg, corresponding to 0.9 mM) [28]. Similar screening of HIS was found in the mixtures of PUT/HIS and CAD/PUT/HIS [II].

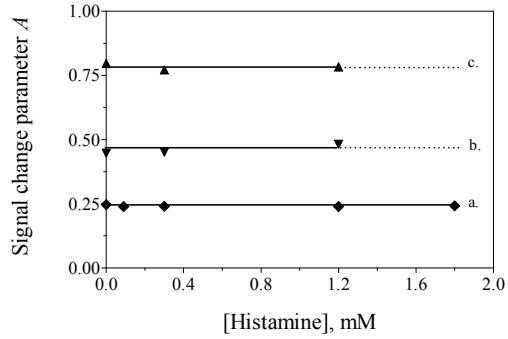


Figure 4. The signal change parameter *A* in the mixtures of CAD and HIS at different concentration rates. HIS concentration is shown on x-axis and CAD concentrations are (a) 0.15 mM; (b) 0.30 mM; (c) 0.60 mM. Measurements were carried out in 0.1 M phosphate buffer (pH 7.0) at 25°C, [PSAO] = 0.108 IU/ml.

So, by applying a PSAO-based biosensor for the detection of HIS or the content of total amines, the results of analysis can be underestimated in case there are several biogenic amines simultaneously present in the sample. The screening phenomenon and the dependence of the total output signal on the rate and relative concentrations of different biogenic amines in the sample can lead to the underestimation of the content of biogenic amines in food, especially if in the course of putrefaction some particular biogenic amines become dominant, such as PUT and CAD in decomposing of white fish.

3.3. Multivariate correlation analysis

As different substrates generate analogous signals, different multivariate models to characterize the impact of these substrates into the resulting signal, enabling the application of the signal parameters for the calibration of a biosensor for several substrates were proposed. This task of multisubstrate calibration inescapably requires signal measurements in solutions with varying concentration ratios of BAs, which can be achieved by addition of a certain amount of definite amine to the probe and so producing a series of BA solutions with variable concentrations.

3.3.1. Hyperbolic model

According to the applied dynamic biosensor model [86], the biosensor signal change parameter A depends hyperbolically on substrate concentration in single substrate solutions:

$$A = \frac{k_{cat}^{*}[E]_{total} c_S^{bulk}}{k_{diff}^{O_2} K_{O_2} K_S + (k_{cat}^{*}[E]_{total} + k_{diff}^{O_2} K_{O_2}) c_S^{bulk}} \tag{4}$$

In Eq. 4 k_{cat}^{*} denotes the apparent catalytic constant of the enzyme-catalyzed reaction, $[E]_{total}$ is the overall concentration of the enzyme in biosensor, $k_{diff}^{O_2}$ is the apparent diffusion constant of oxygen, K_{O_2} is the dissociation constant for the enzyme-oxygen complex, K_S is dissociation constant for the enzyme-substrate complex and c_S^{bulk} is substrate concentration in solution.

As the reaction mechanism for competing BAs is similar, the sum of three hyperbolas was used, each describing the impact of individual substrates, which after appropriate substitutions has the following form:

$$A = \frac{a_1 x}{b_1 + (a_1 + c_1)x} + \frac{a_2 y}{b_2 + (a_2 + c_2)y} + \frac{a_3 z}{b_3 + (a_3 + c_3)z} \tag{5}$$

where *x, y* and *z* are the variables denoting the concentrations of CAD, PUT and HIS accordingly [III], $a_1 - a_3$, $b_1 - b_3$ and $c_1 - c_3$ have the following physical meanings:

$$a = k^*_{cat}[E]_{total} \tag{6}$$

$$b = k^{O_2}_{diff} K_{O_2} K_S \tag{7}$$

$$c = k^{O_2}_{diff} K_{O_2} \tag{8}$$

Applying Eq. 5 as a model for the biosensor signal change parameter, a good correlation with the experimental results (112 measurements in solutions with different concentration of cadaverine, putrescine and histamine) with standard deviation σ = 0.097 and square of the correlation coefficient R^2 = 0.871 was obtained. The values of coefficients of the model (Eq. 5) for the mixtures of different amines are given in Table 4 [III].

Table 4. The values of the calculated coefficients, standard deviation σ and square of the correlation coefficient R^2 for hyperbolic model of the BAs biosensor.

Model	**Coefficient values**	**σ**	**R^2**
$A = \frac{a_1 x}{b_1 + (a_1 + c_1)x} + \frac{a_2 y}{b_2 + (a_2 + c_2)y} + \frac{a_3 z}{b_3 + (a_3 + c_3)z}$	a_1=1.57 b_1=0.39 c_1=0.26 a_2=1.42 b_2=0.41 c_2=0.86 a_3=0.26 b_3=2.06 c_3=10.73	0.097	0.871

The correlation of the calculated and experimental values of biosensor signal change parameter *A* is graphically shown on Fig. 5, where the ideal correlation is shown with a dottled line.

It can be seen that the calculated (using hyperbolic model) values of signal change parameter *A* correlate normally with the experimental data and there are no systematic drifts, except in case of very low reaction effects.

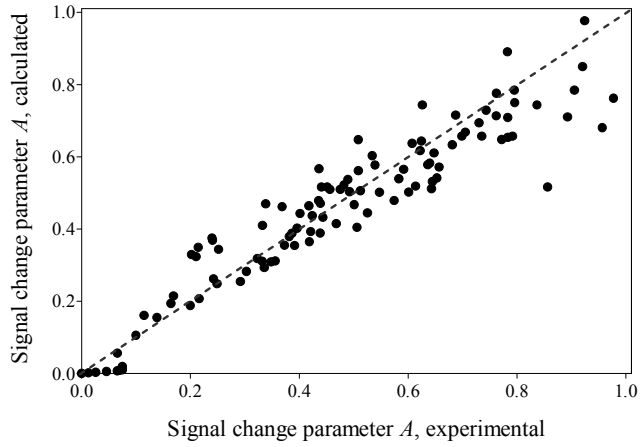


Figure 5. Correlation of the values of maximum signal change parameter *A*, calculated with the help of the hyperbolic model using the experimental data. Dottled line marks the ideal correlation (x = y).

The basic problem with this approximation was the large number of coefficients, requiring at least 9 measurements in different BA mixtures (the original sample and 8 additional solutions, where a definite amount of one or more substrates has been added) for the biosensor calibration. Although it enables the calibration of biosensors in mixtures of several substrates, this procedure can lead to notable experimental noise.

3.3.2. Quantitative concentration – signal relationship

In addition, some more formal models were studied to characterize the relationship between the resulting biosensor signal change parameter *A* and the concentrations of different substrates. These models included different addends as "descriptors" of reactions, going on in the biosensor, taking into account the individual concentrations of BAs and the interference phenomena (inhibition by a competing substrate) of these compounds, which was described with 3 different types of "descriptors", such as the products of duplicate substrate concentrations along with the quadrates and exponents of each single BA concentration:

$$A = ax + by + cz + dxy + fxz + gyz + hx^2 + jy^2 + kz^2 + le^x + me^y + ne^z + p \quad (9)$$

In Eq. 9 *x*, *y* and *z* are the variables denoting the concentrations of different BAs, coefficients *a, b...n* denote the impact of each "descriptor" and *p* is the constant term. These quantitative concentration signal relationships are analyzed in detail in paper [III].

3.4. Immobilization of pea seedling diamine oxidase and the stability of the immobilized enzyme

To warrant the repeated use and operational stability of biosensing systems, which is of crucial importance, biorecognition agents are commonly attached to some kind of insoluble carrier. For the immobilization of diamine oxidase several possibilities of covalent binding (incl. multipoint immobilization) onto nylon-6,6 threads were studied. Nylon-6,6 is a macromolecular compound with cross-linked amide bonds which are unable to bind enzymes directly; thus the activation of nylon threads is essential. The important advantage of nylon-6,6 polymer is its high hydrophilicity and mechanical strength [87].

The activity of the immobilized enzyme was evaluated against the activity of the soluble PSAO on the basis of the signal change parameter *A* of CAD oxidation reaction. The signal change parameters of the soluble and immobilized enzyme were compared for sensors comprising the same amount of nylon thread to eliminate the diffusion effects from the sensor output. In the case of soluble PSAO, the calculated signal change parameter was linearly dependent on the activity of the enzyme with the slope 5.69 ± 0.05 IU/ml (Fig. 6) [IV].

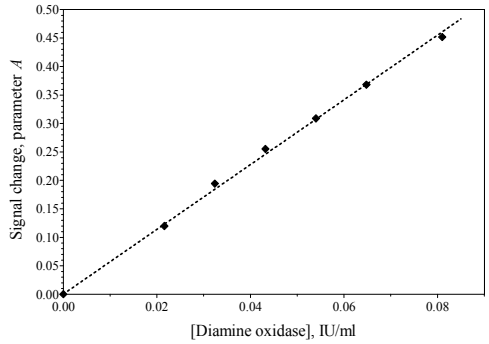
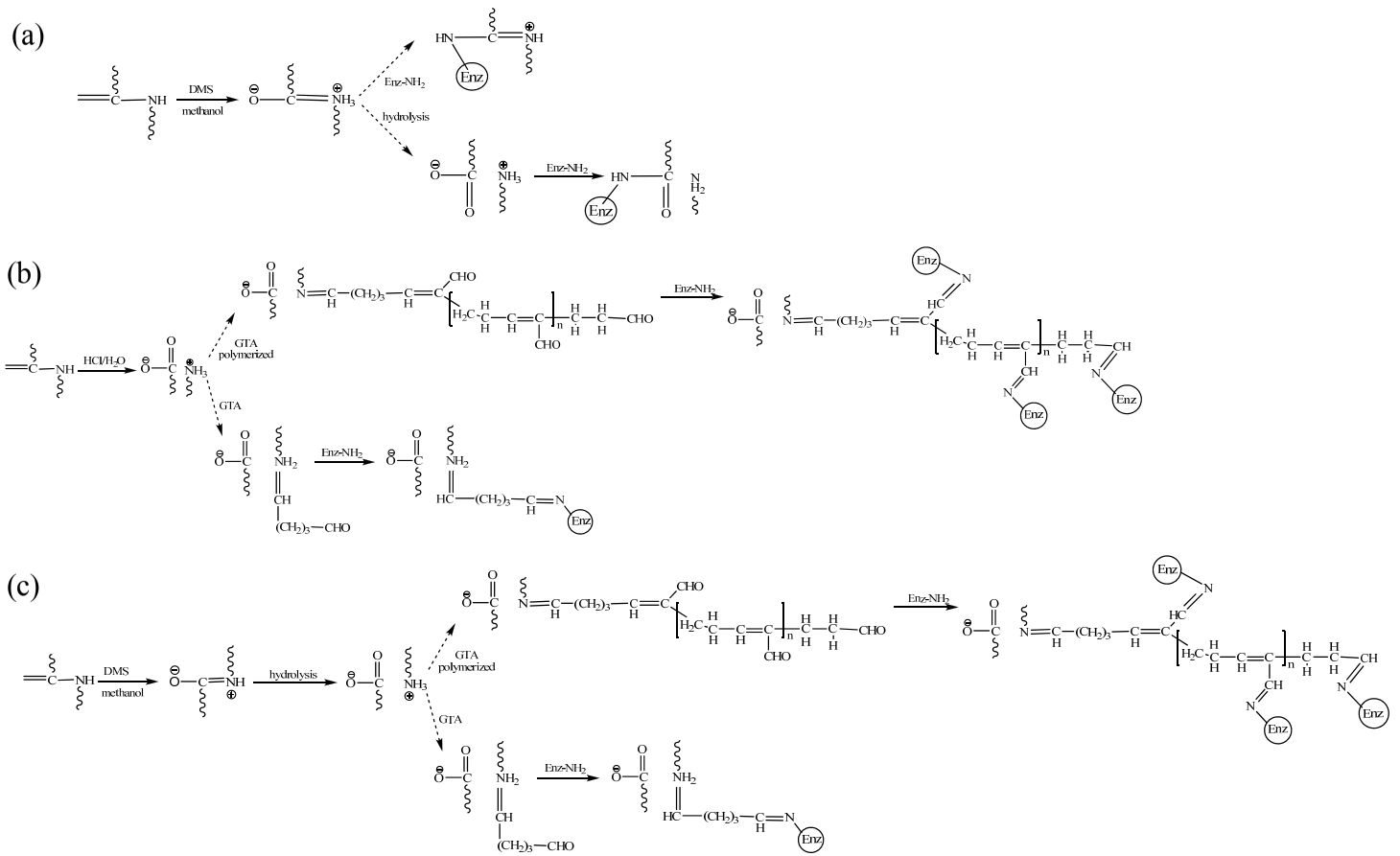


Figure 6. The dependence of total signal change parameter *A* on the activity of soluble PSAO. Measurements were carried out in 1.05 mM CAD solutions in air-saturated 0.1 M phosphate buffer (pH 7.0) at 25°C.

Based on this dependence the specific activity of the immobilized PSAO-containing threads in the units of length (IU/cm) was determined, assuming that the immobilized enzyme was distributed evenly along the thread.

Three different nylon activation methods (the reagents and activation conditions were modified) were used to study the options of effective immobilization of PSAO onto nylon threads. The reaction schemes of the activation of nylon-6,6 are depicted in Scheme 3.



Scheme 3. Immobilization of diamine oxidase onto nylon-6,6, activated with (a) dimethyl sulfate, (b) glutaraldehyde and (c) dimethyl sulfate and glutaraldehyde.

In the first order, dimethyl sulfate (DMS) as an *O*-alkylating agent was used to activate the nylon surface for enzyme immobilization (Scheme 3, a). The yielding imidoesters are highly sensitive towards hydrolysis, leading to their decomposition to corresponding esters and amines and loss of reactivity towards free amines [88,89]. This hydrolysis was minimized by using neutral conditions (pH 7.0) for enzyme incubation. Experiments with DMS were carried out using different nylon activation times (30 to 120 sec with the step of 10 sec) and temperatures (30°C to 60°C with the step of 10°C). The maximum catalytic activity of immobilized PSAO was achieved by applying 40 sec of nylon activation time. Activation at higher temperatures gave threads with proportionally higher PSAO activity; however, the activation of nylon at higher temperatures than 40°C worsened the mechanical properties of the thread. The initial activity of the enzyme-containing thread, obtained by activating nylon for 40 sec at 40°C with DMS, was quite high: $(5.3 \pm 1.0) \times 10^{-4}$ IU/cm. Unfortunately, the inactivation of the immobilized enzyme was very quick: 50% of its initial activity was lost in 24 hours (Fig. 7, a) [IV]. Activating nylon-6,6 surface with DMS, most of the initially bound PSAO was apparently attached to the surface by physical adsorption and not immobilized covalently to the carrier. The activity of these threads decreased with every measurement, although the threads were carefully washed after incubation in the enzyme solution to remove the unbound enzyme. The activity of the immobilized PSAO stabilized on the level of 25% of the initial activity.

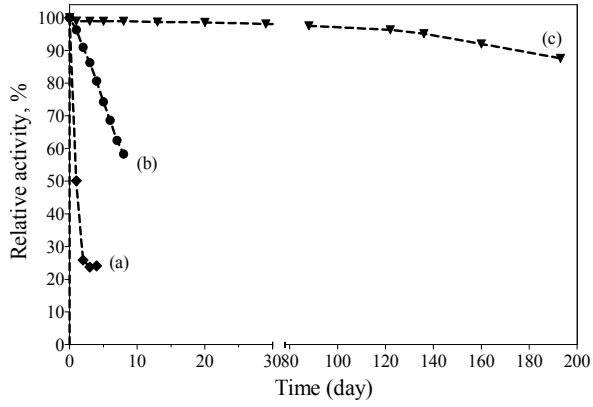


Figure 7. Stability of PSAO-containing nylon threads, activated with: (a) dimethyl sulfate; (b) glutaraldehyde and (c) dimethyl sulfate and glutaraldehyde. Measurements were carried out in 1.05 mM CAD solutions in air-saturated 0.1 M phosphate buffer (pH 7.0) at 25°C. The length of the PSAO-containing threads, coiled around the cathode of sensor, was 90 cm.

In order to increase the amount of potentially reactive centres the polymeric material was subjected to partial acidic hydrolysis. This approach is based on the partial hydrolysis of amide groups (–CO–NH–) leading to the increase in the yield of free amino and carboxyl groups on the polymer surface. Both the carboxyl and amino groups of the partially hydrolyzed nylon can be used for the direct attachment of the enzyme molecules [90]. The greater number of carboxyl groups corresponds to major number of amino groups that can react with enzyme. However, the acid concentration, reaction temperature and the duration of reaction influenced not only the concentration of carboxyl groups, but also the mechanical properties of material. Our immobilization procedure also included the application of glutaraldehyde (GA) as a crosslinking agent in order to increase the distance between the enzyme molecule and the surface of nylon, consequently to obtain higher conformation flexibility for the enzyme. Carrying out the crosslinking reaction in alkaline conditions, monomeric GA polymerized and the forming linker between the surface and enzyme molecule had different forms and length, as the proximal aldehyde group of each GA molecule could link in the classical Schiff´s base fashion with the amine groups of the spacer molecules, leaving the distant aldehyde group available for covalent enzyme coupling. The possible crosslinking reactions between nylon, glutaraldehyde and enzyme are described in Scheme 3, b.

Activating hydrolyzed nylon surface with GA, the initial specific activity of the threads at optimal thread activation conditions (1 h of incubation in 12.5% GA solution in 0.1 M phosphate buffer (pH 7.0) at room temperature) was a bit smaller $(4.4 \pm 1.8) \times 10^{-4}$ IU/cm than with the previous method, but the speed of the inactivation of the immobilized enzyme was considerably lower: 50% of the initial activity was lost in 9 days (Fig. 7, b). Still the inactivation of the immobilized PSAO was too fast for the application of these PSAO-containing threads in biosensors, so the stability of these threads after the activity had dropped below 50% of the initial activity was not followed any further [IV].

In the next step the activity and stability of immobilized PSAO in case nylon threads were consecutively treated with DMS and GA were studied (Scheme 3, c). The specific activity of PSAO-containing threads, achieved with this method was similar to the 2nd method, where the threads were activated with GA. Activating the nylon surface with DMS/GA produced the most stable immobilized PSAO, as multipoint covalent attachment was apparently achieved, which was controlled by washing the threads with 0.1 M NaCl solution (in 0.1 M phosphate buffer, pH 7.0). These threads showed no loss of activity during 3 months, when stored in 0.1 M phosphate buffer (pH 7.0) at 4°C. After that date, the activity began to drop slowly. The calculated half-life of these threads was over 400 days. In continuous everyday operation, the activity of the threads began to decrease gradually after 30 days or 120 measurements (Fig. 7, a), as it was determined in repeated studies at similar CAD concentrations and during these studies any inhibiting effect of H_2O_2, produced in the course of CAD oxidation, were not detected [IV].

The summarized data of the initial activities and stabilities of the PSAO-containing threads, prepared with different methods of activating the nylon surface, are shown in Table 5.

Table 5. The specific activity and stability of immobilized with different methods PSAO.

Immobilization method	Specific activity × 10^4 (IU/cm)*	Inactivation half-life
Nylon-6,6 activated with DMS	5.3 ± 1.0	24 hours
Nylon-6,6 activated with GTA	4.4 ± 1.8	9 days
Nylon-6,6 activated with DMS/GA	4.6 ± 0.6	Over 400 days

* The specific activity of PSAO was measured towards 1.05 mM cadaverine.

For the characterization of the catalytic properties of the immobilized enzyme, the K_M values for soluble and immobilized on DMS/GA activated nylon PSAO were calculated. For the immobilized enzyme, the value of K_M decreased approximately 2.5 times in comparison with the soluble enzyme, being respectively 0.60 ± 0.06 mM and 1.41 ± 0.17 mM. As the K_M value determines the sensitivity and working range of biosensing systems, this change of the K_M value during immobilization should be considered when applying the immobilized PSAO for practical purposes.

3.5. Application of diamine oxidase-based biosensor for the assessment of white fish quality and validation of biosensor results

Using activated threads with the length of 30 to 130 cm, it was possible to provide a layer of immobilized PSAO with the total enzyme activity ranging from 0.014 to 0.060 IU. The PSAO-containing threads formed a biocatalytic spiral, which density of coiling determined also the diffusion properties of the biosensor enzyme-containing matrix. So, both the total activity and diffusion parameters of the biosensor enzyme-containing layer varied, determining together the sensitivity of the biosensor [87]. Threads of 130 cm covered approximately 100% of the cathode surface of the oxygen sensor and formed the most dense possible diffusion barrier; at the same time the amount of PSAO in these threads was the highest.

The hyperbolic dependences of the values of the signal change parameter A on CAD concentration for biosensors comprising 30 to 130 cm PSAO threads are shown on Fig. 8. Good assay reproducibility was obtained as reflected by the average response for at least 3 repeated analyses with standard errors 0.0001 to 0.001. The reproducibility of fabricating diamine oxidase immobilized nylon

thread was also assessed by preparing several threads under the same conditions. The relative standard error was 0.01 ($n = 3$), showing the high reproducibility of the fabrication process [V].

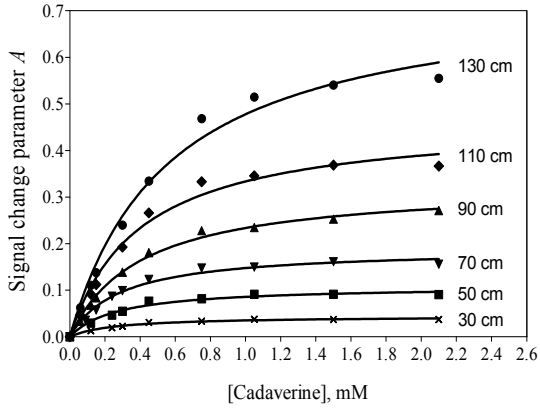


Figure 8. The dependence of biosensor signal change parameter A on the concentration of CAD for sensors with threads of different length (PSAO activity $(4.58 \pm 0.04) \times 10^{-4}$ IU/cm). All measurements were carried out in air-saturated 0.1 M phosphate buffer solutions (pH 7.0) at constant mixing at 25°C. Experimental points are the mean of 3 measurements.

The curves in Fig. 8 were characterized with 2 independent coefficients: $K_{1/2}$ is the half-signal change constant and A_{max} is the theoretical maximum of signal change for a particular biosensor. These values for biosensors, comprising different amounts of PSAO threads, are given in Table 6.

$K_{1/2}$ was the basic value for the estimation of the determination range of these biosensors and based on the biosensor signal change curves, the biosensor sensitivity as the ratio $S = A_{max}/K_{1/2}$ was calculated. The sensitivity of biosensors with 130 cm long PSAO threads was nearly 8 times higher than the sensitivity of biosensors with 30 cm long threads, ranging from 1.324 to 0.168 mM^{-1}, accordingly. At the same time, the value of A_{max} increased more than 16 times. The value of $K_{1/2}$ increased 2 times along with the increase of the length of the thread in studied systems, so the determination range was wider for biosensors with longer threads – being from 0.01 to 1.13 mM of CAD.

Table 6. Characteristics of calibration curves of biosensor with different length of diamine oxidase-containing threads (PSAO activity was $(4.58 \pm 0.40) \times 10^{-4}$ IU/cm).

Length of the thread (cm)	$K_{1/2}$[(a)] (mM)	A_{max}[(a)]	Sensitivity[(b)] (mM^{-1})	Range of cadaverine detection[(c)] (mM)
30	0.263 ± 0.037	0.044 ± 0.002	0.168	0.075–0.526
50	0.274 ± 0.045	0.109 ± 0.005	0.398	0.033–0.548
70	0.287 ± 0.037	0.190 ± 0.007	0.661	0.018–0.574
90	0.405 ± 0.036	0.328 ± 0.010	0.809	0.015–0.811
110	0.401 ± 0.058	0.467 ± 0.023	1.165	0.011–0.802
130	0.564 ± 0.080	0.747 ± 0.041	1.324	0.009–1.128

[(a)] $K_{1/2}$ and A_{max} have been determined by fitting calibration curves to the hyperbole $A = A_{max}[\text{Cad}] / (K_{1/2} + [\text{Cad}])$;
[(b)] the sensitivity of the sensor is presented as the ratio $A_{max}/K_{1/2}$;
[(c)] lower limit of detection range was determined as a substrate concentration corresponding to 5% of A_{max} value for a particular biosensor and upper limit as $2K_{1/2}$.

Based on the obtained data, the biosensor sensitivity increased linearly along with increase of the length of the thread and the amount of the immobilized PSAO with the slope 0.0107 ± 0.0006 $mM^{-1}cm^{-1}$ ($R^2 = 0.9915$) (Fig. 9) [V].

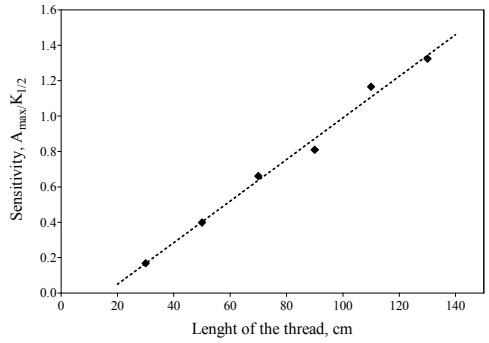


Figure 9. The dependence of biosensor sensitivity on the length of PSAO-containing thread.

Based on the biosensor sensitivity studies and the average levels of CAD and PUT in white fish, a biosensor with 90 cm PSAO-containing thread was used for the evaluation of the content of biogenic amines in hake probes during their preservation at 4°C [V].

The PSAO-based biosensor signal change parameter *A* reflected the combined concentration of CAD and PUT, as HIS was totally screened by these compounds [II]. From the biosensor signal change parameter the effective amount of CAD (a combined value of CAD and PUT) according to the

calibration curve was calculated (Fig. 10). The slope of the linear part of the calibration curve of this biosensor was (0.46 ± 0.02) (R^2 = 0.983).

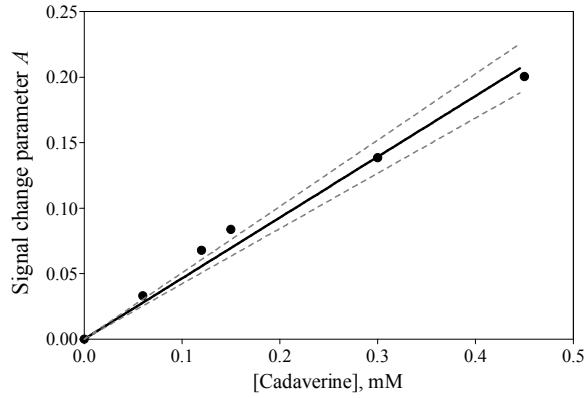


Figure 10. The linear dependence of biosensor signal change parameter *A* on the concentration of CAD for sensor with 90 cm of PSAO-containing thread (PSAO activity (4.58 ± 0.04) × 10^{-4} IU/cm). The correlation is shown in 95% confidence intervals. All measurements were carried out in air-saturated 0.1 M phosphate buffer solutions (pH 7.0) at constant mixing at 25°C. Experimental points are mean of 3 measurements.

The effective amount of CAD rose slowly within the first 36 hours. After that, the putrefaction process speeded up and the effective CAD concentration reached the upper limit of the biosensor working range on the 5th day of hake storage (Fig. 11) [V].

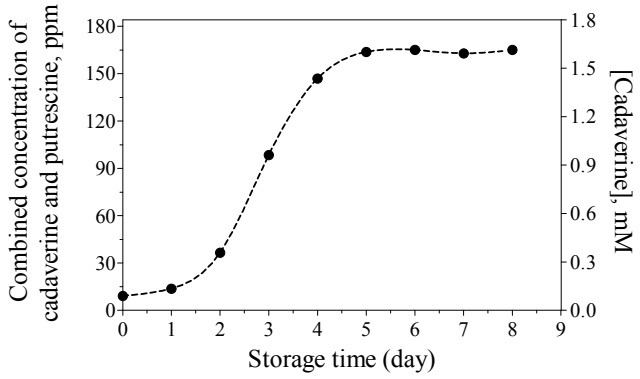


Figure 11. The amount of CAD and PUT in hake probes stored at 4°C, measured with biosensor, comprising 90 cm of PSAO-containing thread (diamine oxidase activity (4.58 ± 0.40) × 10^{-4} IU/cm). All measurements were carried out in air-saturated 0.1 M phosphate buffer solutions (pH 7.0) at constant mixing at 25°C. Experimental points are the mean of 3 measurements.

The results, obtained with the biosensor were compared with HPLC studies, where CAD, PUT and HIS contents in hake probes were determined separately. The HIS content in all studied hake probes (by HPLC) was found to be under the allowed MRL of 50 ppm; its maximum at 41 ppm was reached on the 6th day of storage. Afterwards the HIS content began to decrease, likely due to the decrease of activity of bacteria producing HIS decarboxylase, which catalyzes the formation of HIS from histidine [91].

The combined concentration of CAD and PUT in hake probes, determined by the HPLC analysis, was increasing gradually day after day from its initial value of 8 ppm to 243 ppm. The combined amounts of CAD and PUT in hake probes, determined by HPLC analysis were in good agreement with biosensor results. The correlation between biosensor and HPLC results was linear till the 5th day of the fish storage, when the biosensor reached the upper limits of its detection range. The slope of this linear correlation between biosensor and HPLC results was found to be (1.05 ± 0.09); showing that the biosensor results were a little overestimated in comparison with HPLC results, but this overestimation was even within the whole biosensor working range (Fig. 12) [V].

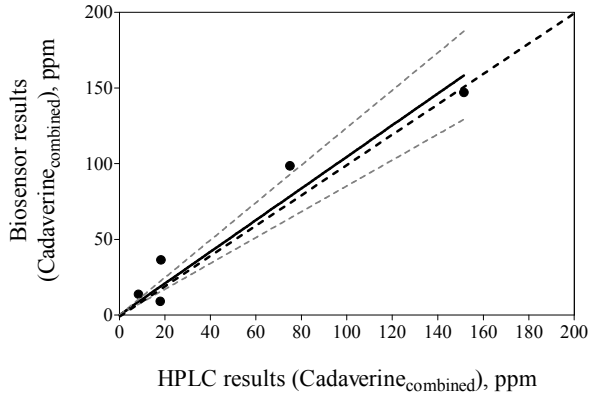


Figure 12. Comparison of the combined concentrations of CAD and PUT (ppm), measured with biosensor and with HPLC. HIS was excluded from the comparison, as it is screened by CAD and/or PUT in biosensor measurements, the correlation is shown in 95% confidence intervals. The ideal correlation ($x = y$) is shown as dottled line.

In addition, we calculated the correlation between the biosensor and HPLC results, when the concentration of HIS was added to the latter. This resulted also in a linear correlation with a considerabely lower value of the slope (0.79 ± 0.04), showing a 20% underestimation of the results by biosensor, which is once again indicating that the detection of HIS in the presence of CAD and PUT with the biosensor is problematic.

4. CONCLUSIONS

Diamine oxidase was isolated and purified from pea seedlings with the aim of using it in biosensors as a biorecognition element for biogenic amines. The specific activity and selectivity of the purified enzyme was studied with an oxygen sensor, following the decay of dissolved oxygen concentration in the course of amine oxidation reaction, catalyzed by the enzyme. The enzyme catalytic characteristics were calculated from the transient signal of the oxygen sensor with the help of a model, proposed earlier for amperometric biosensors. The isolated pea seedling amine oxidase (PSAO) had the highest selectivity towards cadaverine and putrescine [I], forming a solid base for the construction of PSAO-based biosensor for a rapid detection of cadaverine and putrescine.

The biosensor response was also studied in the mixtures of several biogenic amines. The interaction of cadaverine and putrescine partially eliminated their individual impacts on the signal, causing the decrease of the resulting signal output, which was not an additive sum of the signals of single substrates and the biosensor response parameters in cadaverine/putrescine mixtures were 1.14 ± 0.02 times smaller than presumed [II]. In the presence of cadaverine and/or putrescine, histamine did not cause any detectable signal (histamine concentration varied from 0.01 to 3.0 mM, exceeding several times the histamine maximum residue level in foods allowed by EEC regulations) [II]. These screening effects should be taken into consideration when analyzing food samples, in which the ratio of different biogenic amines is different and changes in the course of protein decomposition [II].

For the examination of individual impacts of the studied substrates into the biosensor output signal, multivariate correlation analysis was used to construct a model enabling the characterization of the biosensor output and biosensor calibration towards several substrates in the mixtures of different biogenic amines. A good correlation of the proposed model with the experimental results (112 measurements in solutions with different concentrations of cadaverine, putrescine and histamine) was established (standard deviation 0.097 and correlation coefficient 0.93) [III].

PSAO was successfully immobilized onto nylon threads by activating nylon surface with dimethyl sulfate and glutaraldehyde before the attachment of the enzyme. The immobilized enzyme was stable for at least 3 months at 4°C or 120 measurements. The specific activity of the immobilized enzyme was $(4.6 \pm 0.4) \times 10^{-4}$ IU/cm, which was sufficient for the construction of biosensors. The value of K_M of the immobilized enzyme was approximately 2.5 times lower in comparison with the soluble enzyme and was 0.6 ± 0.06 mM [IV].

A simple biosensor, comprising exchangeable PSAO-activated nylon threads with the length of 90 cm and an oxygen sensor was used for the detection of biogenic diamines. With this biosensor we could successfully evaluate the quality of hake probes, preserved for several days at 4°C, within 5 minutes and the obtained results were in good correlation with the outcome of HPLC analy-

ses. The applied flexible setup of a biosensor enabled to vary the determination range and sensitivity of the system, as well to renew the enzyme-containing biorecognition element easily and repeatedly within 1–2 minutes [V].

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SUMMARY IN ESTONIAN

Diamiini oksüdaasil baseeruvad biosensorid: konstrueerimine ja tööpõhimõtted

Käesoleva doktoritöö eesmärgiks oli uurida võimalusi biogeensete amiinide biosensori konstrueerimiseks, kasutades bioselektiivse elemendina herneidudest (*Pisum sativum*) eraldatud diamiini oksüdaasi (PSAO) ja baaselektroodina hapnikuandurit [I–V].

Biogeensed amiinid on aminorühma sisaldavad ühendid, mis tekivad enamasti aminohapete dekarboksüleerimisprotsessi tulemusena. Biogeensete amiinide avastamine on oluline mitte ainult nende toksilisuse seisukohast, vaid ka seetõttu et neid saab kasutada indikaatoritena vaske sisaldavate toiduainete värskuse ja kvaliteedi hindamisel. Olulisemad biogeensed amiinid, mida kasutatakse indikaatoritena on histamiin, kadaveriin ja putrestsiin. Praegusel hetke on kehtestatud maksimaalne lubatud määr toidus vaid histamiini jaoks, kuigi mitmed viimaste aastate uuringud on näidanud ka teiste biogeensete amiinide soovimatuid efekte inimeste tervisele. Biogeensete amiinide määramiseks kasutatakse tavapäraselt vedelkromatograafilisi meetodeid. Üheks võimalikuks alternatiiviks traditsioonilis-tele määramismeetoditele on biosensorite kasutamine – nende eeliseks on analüüsi odavus, kiirus ja lihtsus. Biosensorite kasutamisel jääb ära aeganõudev proovide ettevalmistamine ning analüüse on võimalik teostada kohapeal.

Biogeensete amiinide biosensorite bioäratundmissüsteemina kasutati puhastatu diamiini oksüdaasi, mis katalüüsib amiinide oksüdeerumist hapniku toimel. Lahustunud hapniku kontsentratsiooni muutumise alusel selle ensüümi poolt katalüüsitud reaktsiooni käigus määrati hapnikuanduri tasakaalueelsest väljundsignaalist ensüümi eriaktiivsus ja selektiivsus erinevate amiinide suhtes, kasutades varem väljatöötatud biosensorite mudelit. PSAO selektiivsus oli kõige kõrgem kadaveriini ja putrestsiini suhtes [I], mis võimaldas konstrueerida PSAO-l põhineva biosensori vastavate biogeensete amiinide kiireks määramiseks.

Biogeensete amiinide koosmõju uurimiseks biosensori väljundsignaalile viidi läbi mõõtmised süsteemides, mis sisaldasid samaaegselt kahte või enamat amiini. Tulemustest selgus, et kadaveriin ja putrestsiin elimineerivad osaliselt üksteise individuaalset mõju väljundsignaalile ning biosensori väljundsignaali parameetrite väärtused olid nende erinevates segudes 1,14 ± 0,02 korda väiksemad kui eeldatud [II]. Histamiini sisaldavate süsteemide korral selgus tulemustest, et kadaveriini ja/või putrestsiini olemasolul ei avalda histamiin biosensori väljundsignaalile detekteerivat mõju (histamiini sisaldus varieerus 0,01 kuni 3,0 mM, mis ületab EEC regulatsioonide kohaselt mitu korda histamiini maksimaalse lubatud piirmäära toidus) [II]. Seega tuleb arvestada erinevate amiinide koosmõju efektidega biosensori väljundsignaalile, kui analüüsitakse toiduainete proove, milles amiinide kontsentratsioonide suhted muutuvad valkude lagunemise käigus [II].

Uuritud substraatide mõju osakaalu kirjeldamiseks biosensori väljundsignaalile kasutati mitme muutujaga korrelatsioonanalüüsi eesmärgiga kontrueerida mudel, mis võimaldab iseloomustada biosensori väljundsignaali ja biosensori kalibreerimist mitme substraadiga süsteemides. Väljapakutud mudeli korral saadi hea korrelatsioon katsetulemuste (112 mõõtmist erinevate kadaveriini, putrestsiini ja histamiini kontsentratsioonide korral) ja mudeli abil leitud tulemuste vahel (standardhälve 0,097 ja korrelatsioonikordaja 0,93) [III].

PSAO stabiliseerimiseks immobiliseeriti see edukalt nailonniidile, mida eelnevalt oli aktiveeritud dimetüülsulfaadi ja glutaaraldehüüdiga. Immobiliseeritud ensüüm oli stabiilne vähemalt 3 kuud 4°C juures või vähemalt 120 mõõtmise jooksul. Immobiliseeritud ensüümi spetsiifiline aktiivsus oli $(4{,}6 \pm 0{,}4) \times 10^{-4}$ IU/cm, mis oli piisav biosensori kontrueerimiseks. Immobiliseeritud ensüümi K_M väärtus oli ligikaudu 2,5 korda väiksem võrreldes lahustunud ensüümiga, ehk vastavalt siis $0{,}6 \pm 0{,}06$ mM [IV].

Konstrueeritud biosensorit, kus hapnikuanduri pinnale oli keritud 90 cm PSAO-sisaldavat niiti, kasutati edukalt 4°C juures säilitatavate hõbeheigi proovide värskusehindamiseks. Eelnevalt töötlemata kalaproovide tulemused saadi 5 minuti jooksul ning need olid heas korrelatsioonis HPLC analüüsil saadud tulemustega. Kasutatud biosensor võimaldas ensüüminiidi pikkuse varieerimise teel muuta biogeensete diamiinide määramise vahemikku ja süsteemi tundlikkust ning uuendada lihtsalt ja kiirelt (1–2 minuti jooksul) süsteemi bioäratundmiselementi [V].

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2005–2007	University of Tartu, *M.Sc*.in colloid and environmental chemistry
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Main scientific publications

1. **Kivirand, K.,** Rinken, T., Purification and properties of amine oxidase from pea seedlings. Proceedings of the Estonian Academy of Sciences Chemistry, 56 (2007) 164–171.
2. **Kivirand, K.,** Rinken, T., Interference of the Simultaneous Presence of Different Biogenic Amines on the Response of an Amine Oxidase Based Biosensor. Analytical Letters, 42 (2009) 1725–1733.
3. **Kivirand, K.,** Rinken, T., Preparation and Characterization of Cadaverine Sensitive Nylon Threads. Sensor Letters, 7 (2009) 580–585.
4. **Kivirand, K.,** Rebane, R., Rinken, T., A Simple Biosensor for Biogenic Diamines, Comprising Amine Oxidase – Containing Threads and Oxygen Sensor. Sensor Letters (2011) (Article in Press).
5. **Kivirand, K.,** Rinken, T., Biosensors for Biogenic Amines: the Present State of Art. Analytical Letters, (2011) (Article in Press).
6. Rinken, T., Rinken, P., **Kivirand, K.** Signal Analysis and Calibration of Biosensors for Biogenic Amines in the Mixtures of Several Substrates. In: Biosensors for Health, Environment and Biosecurity, InTech – Open Access Publisher (2011) (In Press).

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Tähtsamad teaduspublikatsioonid

1. **Kivirand, K.,** Rinken, T., Purification and properties of amine oxidase from pea seedlings. Proceedings of the Estonian Academy of Sciences Chemistry, 56 (2007) 164–171.
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6. Rinken, T., Rinken, P., **Kivirand, K.** Signal Analysis and Calibration of Biosensors for Biogenic Amines in the Mixtures of Several Substrates. In: Biosensors for Health, Environment and Biosecurity, InTech – Open Access Publisher (2011) (Trükis).

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